

GROWTH, YIELD AND PHYSIOLOGICAL RESPONSES OF THE BIOENERGY CROP
Miscanthus × giganteus TO FERTILIZER, BIOCHAR AND DROUGHT

A Thesis
by
ALYSSA LEIGH TEAT

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Abstract

GROWTH, YIELD AND PHYSIOLOGICAL RESPONSES OF THE BIOENERGY CROP *Miscanthus × giganteus* TO FERTILIZER, BIOCHAR AND DROUGHT

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As atmospheric greenhouse gas concentrations continue to rise due to the burning of nonrenewable fossil fuels, there is a growing need for cleaner and renewable fuel sources. *Miscanthus × giganteus*, or giant miscanthus, has emerged as a potential high-yielding bioenergy crop with the ability to grow in marginal soils that often have low nutrient and water availability. However, if precipitation is reduced by future climate change, then enhanced drought may constrain the yields of giant miscanthus.

The goals of this study were to determine whether giant miscanthus could be established, grown, and produce yields in the Western North Carolina mountains comparable to those in other studies, and also to determine whether fertilization and biochar soil additions could improve growth and yield. At two field sites, Mills River, NC (35°25'24" N, 82°33'33" W, 650 m) and Valle Crucis, NC (36°11'49" N, 81°47'09" W, 830 m), fertilizer and biochar treatments were applied in a randomized block design (fertilizer at 0 and 100 kg NPK ha⁻¹ and biochar at 0 and 15 t ha⁻¹). Rhizomes were planted in spring of 2012 and allowed to grow for two full growing seasons. No significant treatment differences were found for photosynthesis or stomatal conductance, but these parameters were significantly higher at Mills River than at Valle Crucis. No biochar or fertilizer effects were found for yield in either site in both 2012 and 2013, though first year yields of giant

miscanthus were higher at Mills River than at Valle Crucis, with average plot yields of 1.65 and 1.00 Mg DW ha⁻¹ respectively. Second year yields were approximately 10 times higher than first year yields, with an average plot yield of 15.57 Mg DW ha⁻¹ at Mills River and 16.95 Mg DW ha⁻¹ at Valle Crucis. The high growth rates, at least for the first two years of growth, show that this perennial bioenergy crop is capable of growing in the Western North Carolina mountains and producing yields comparable to those in other areas where this crop has been grown.

A second study was conducted to understand how giant miscanthus responds physiologically to short-term drought and whether biochar can alleviate drought stress by improving soil water status. Giant miscanthus rhizomes were grown for 75 days in either soil-only or soil amended with biochar at a rate of 50 t ha⁻¹. Drought was induced in half of the plants by withholding watering and physiological measurements (gas exchange, plant water potential, chlorophyll fluorescence) were made every three days throughout the drought period and during a rewatering event. Biochar at the rate applied did not alleviate drought stress and it increased soil water status only under well-watered conditions. Although soil volumetric water content began to decrease immediately with lack of watering, most physiological parameters, including leaf water potential, photosynthetic rates and stomatal conductance, did not decrease until water content had reached 38% of well-watered levels, 12 days after drought was induced. After rewatering, leaf water potential recovered to within 90% of pre-drought rates within one day while gas exchange required three days to recover. No differences were seen in total aboveground biomass between plants grown with or without biochar, despite higher soil water status prior to drought, nor was there any effect of drought. The lack of effects was likely due to the short duration of the treatment. The ability of giant miscanthus to recover quickly suggests that the impacts of short-term drought can be eliminated rapidly, which may contribute to its ability to grow productively in marginal soils.

Acknowledgments

I'd like to first and foremost thank my advisor, Dr. Howie Neufeld, for deciding to take me on as a graduate student and for all of his support throughout these past three years. He really harnessed my love for plant physiology and I couldn't have asked for a better mentor. I'd also like to thank my committee members Dr. Ron Gehl, for all of his help regarding growing *Miscanthus* and maintaining my field site in Asheville, and Dr. Mike Madritch, for his help in editing this thesis and carbon and nitrogen analysis. I am grateful for Dr. Eva Gonzales for assistance in getting this project off the ground. Phillip Jennings graciously donated all of the *Miscanthus* and biochar used in this project. Dr. Josh Heitman at NCSU analyzed my soils for their water retention. Tom Eschelman at the Valle Crucis Conference Center allowed us to use their land and plant *Miscanthus* there. Thanks to everyone in the Neufeld lab for helping with field work when I needed it, my herbarium family for all the moral support, and all of my fellow graduate students who have helped me countless times. But I am especially thankful for Patrick Sullins for his unconditional support throughout this process.

Funding for this project came from Sigma Xi's Grant-in-Aid of Research program, and Appalachian State University's Office of Student Research, Graduate Student Association, and Research Institute for Environment, Energy, and Economics.

Dedication

I would like to dedicate this thesis to my family for all their support throughout the years and being there for me.



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Foreword

The experiments presented in chapters 1 and 2 of this thesis have been formatted as two separate, publishable papers with the intention of submitting them to scientific journals. Chapter 1 is focused in the field portion of the research while chapter 2 is focused on the greenhouse portion. References have been formatted to follow the style of the scientific journal New Phytologist.

Chapter 1: Growth and yield of *Miscanthus × giganteus* grown in fertilized and biochar-amended soils in the Western North Carolina mountains

Abstract

Miscanthus × giganteus, or giant miscanthus, is a perennial C₄ grass currently being grown worldwide for bioenergy production; however, there is concern about whether giant miscanthus is capable of producing high yields when grown in marginal soils in a temperate climate. The goals of this study were to determine whether giant miscanthus could be established, grown, and produce yields in the Western North Carolina mountains comparable to those in other studies, and also to determine whether fertilization and biochar soil additions could improve growth and yield. Study sites were located at Mills River, NC (35°25'24" N, 82°33'33" W, 650 m) and Valle Crucis, NC (36°11'49" N, 81°47'09" W, 830 m). Treatments consisted of two levels of fertilizer application (0 and 100 kg N ha⁻¹) and two levels of biochar application (0 and 15 t ha⁻¹), with three replicates per treatment combination in a fully randomized block design at each site. Rhizomes were planted in spring of 2012 and allowed to grow for two full growing seasons. Gas exchange measurements were made in the first year before harvesting all aboveground biomass, and biomass was harvested again in the second year. No treatment effects were found for photosynthesis or stomatal conductance, but these parameters were greater at Mills River than at Valle Crucis. No biochar or fertilizer effects were found for yield in either site regardless of the treatments applied, though first year yields of giant miscanthus were greater at Mills River than at Valle Crucis, with average plot yields of 1.65 ± 0.140 and 1.00 ± 0.122 Mg DW ha⁻¹ respectively. First year overwinter survival rates of rhizomes were 100% for both field sites. Second year yields in 2013 were not significantly different between field sites but were approximately 10 times greater than those in the first year, with an average plot yield of

$15.57 \pm 0.716 \text{ Mg DW ha}^{-1}$ at Mills River and $16.95 \pm 1.033 \text{ Mg DW ha}^{-1}$ at Valle Crucis. The high survival and growth rates, at least for the first two years of growth, show that this bioenergy crop is capable of growing in the Western North Carolina mountains and producing yields comparable to those in other areas of the country where this crop has been grown.

Introduction

As atmospheric greenhouse gas concentrations continue to rise due to the burning of nonrenewable fossil fuels, there is a growing need for cleaner and renewable fuel sources. Bioenergy crops have long been promoted as alternatives to fossil fuels because these crops burn cleaner and potentially can reduce GHG emissions (Hill *et al.*, 2006; Wang, M *et al.*, 2012), although this potential is disputed (Fargione *et al.*, 2008; Searchinger *et al.*, 2008). Despite the potential benefits, bioenergy crop production has yet to become competitive with fossil fuel use due to a lower energy conversion efficiency of the bioenergy system (Hattori & Morita, 2010). One of the most important issues surrounding bioenergy production is the displacement of essential food crops by bioenergy crops, which can result in higher food prices in some circumstances and potentially lower food security in the long run (Hattori & Morita, 2010; Ra *et al.*, 2012). If bioenergy crops can produce high yields in marginal soils that are not currently used for food production, some limitations to growing bioenergy plants can be avoided. In addition, such plants should be able to produce high yields with minimal inputs in order to reduce costs and to remain sustainable in the long run (van der Weijde *et al.*, 2013).

Perennial rhizomatous grasses have emerged among the top candidates for bioenergy production due to their high productivity and extended life span, often as long as 10-20 years (Jing *et al.*, 2012). Because many of these species also invest large amounts of resources into belowground biomass (VanLooke *et al.*, 2012) and relocate their nutrients to their rhizomes each winter, they tend to have high nutrient-use efficiencies, which reduces their need for extensive fertilization (Heaton *et al.*, 2008). Additionally, there is no need to till soils each year with perennial rhizomatous grasses, which reduces soil erosion and nutrient leaching while concomitantly enhancing carbon sequestration in the soil (Tonini *et al.*, 2012).

Miscanthus × giganteus Greef et Deu (Greef & Deuter, 1993), hereafter giant miscanthus, has received attention over the past few decades because it is high yielding under a variety of climatic conditions (Lewandowski *et al.*, 2000) with a low risk of invasiveness due to its sterility, unlike its

parent species *M. sinensis* (Jørgensen, 2011). Some of its high yield capabilities may be due, in part, to its C₄ photosynthetic pathway. C₄ species are generally better at accumulating carbon under hot, dry, sunny conditions and have much greater water and nitrogen use efficiencies (Brown, 1978) than C₃ species, which allows them to achieve higher yields under certain environmental conditions, such as when soils are dry or of low nutrient content. Under optimal conditions, giant miscanthus has been reported to obtain yields of up to 30 Mg DW ha⁻¹ (Lewandowski *et al.*, 2000).

Despite promise as a biofuel crop, if giant miscanthus is to be grown in marginal soils, then rhizome establishment and overwinter survivorship in colder climates must achieve high rates of success (Clifton-Brown & Lewandowski, 2000). Even if there is successful establishment and survival, productivity may still be constrained by a lack of available nutrients in poor soils, and despite high nitrogen use efficiency, giant miscanthus might still require the use of fertilizers, which entail additional energy costs. Responses of giant miscanthus to fertilization are variable, ranging from no response (Christian *et al.*, 2008; Maughan *et al.*, 2012) to significant increases in yield and productivity (Boehmel *et al.*, 2008; Wang, D *et al.*, 2012) when applied at very high levels. These conflicting results are likely due to differences in growing conditions and inherent differences in soil fertility among the various studies. The presence of an N-fixing bacterium may also help giant miscanthus meet its nitrogen demands, especially if growing on low N soils (Eckert *et al.*, 2001; Davis *et al.*, 2010).

One possible way to increase soil quality, and perhaps nutrient status, but without the added costs of fertilization, may be through the use of biochar. Biochar is a charcoal-like, black carbon compound produced during pyrolysis, a process in which organic matter is heated to temperatures between 400°C and 600°C in the absence of oxygen. Generally, biochar is characterized by a high C:N ratio (Nelson *et al.*, 2011) and can contain macro and micronutrients, including N, P, K, Ca, Mg, S (Graber *et al.*, 2010). When added as an amendment to soils, biochar can alter soil texture, porosity, and density (Atkinson *et al.*, 2010). The highly porous nature of biochar (Fig. 1) can also increase the

water holding capacity of soils (Kammann *et al.*, 2011), which will increase the amount of available water to plants and alleviates drought stress in arid climates.

While all of these soil benefits provided by biochar often lead to increases in plant productivity and yield (Biederman & Harpole, 2013), it remains unclear whether biochar can be cost effective and beneficial when applied on a larger agricultural scale (Jones *et al.*, 2012). There are also important disadvantages to biochar-amended soils, which may have implications for large-scale field studies. Beesley and Dickinson (2011) reported that the significant increase in dissolved organic carbon in soils with biochar amendment led to increased copper and lead mobility. Other studies have shown decreased NO₃ availability due to immobilization (Nelson *et al.*, 2011) and decreased plant growth resulting from lower N availability (Deenik *et al.*, 2010). Biochar-amended soils also exhibit less nitrate leaching, which is generally beneficial, but also suggests N immobilization and therefore reduced N available for plant uptake (Bell & Worrall, 2011).

Because of the high variability in results, in part due to differences in the type of biochar used, application rate, and soil type, a broader understanding of the effects of biochar on soils is still necessary. Few studies have documented the impact of biochar on plant physiological responses, and even fewer studies have grown giant miscanthus in biochar-amended soils. The goals of this study were to determine whether giant miscanthus could be established, grown, and produce yields in the Western North Carolina mountains comparable to those in other studies, and also to determine whether fertilization and biochar soil additions could improve growth and yield.



Figure 1. Scanning electron micrograph of a piece of biochar. Photo by author.

Methods

Field sites

Two field sites of differing elevations were used for this study: Mills River, NC (35°25'24" N, 82°33'33" W) is a lower elevation site (650 m), while Valle Crucis, NC (36°11'49" N, 81°47'09" W) is higher (830 m). Both sites had been previously cultivated with a variety of crops and, prior to this study, both sites had been recently tilled.

Climate information, including total monthly precipitation and average monthly maximum and minimum air temperatures, was collected using the nearest weather station data (State Climate Office of North Carolina). The Fletcher weather station is located at the Mills River site, while the closest station to the Valle Crucis site is the Boone weather station (12.2 km away, 210 m higher).

Experimental setup

The experiment consisted of four treatments, fertilizer (0 and 100 kg NPK ha⁻¹) and biochar (0 and 15 t ha⁻¹) with 3 replicates per treatment combination in a fully randomized block design at each site, for a total of 12 plots, 2 x 2 m each (Fig. 2). Fertilizer was applied using Scotts Osmocote 14-14-14 slow release fertilizer (The Scotts Company, Marysville, OH). Biochar was obtained from Range Fuels (Soperton, GA) and was produced from pine chips, although the production temperature is unknown. Both fertilizer and biochar were surface broadcast on the appropriate plots and tilled in to a depth of 20 cm using a mechanical tiller to mix thoroughly into the soil prior to planting.

Genetically identical giant miscanthus rhizomes, obtained from Repeve Renewables (Soperton, GA), were planted on May 26, 2012, at Valle Crucis and on June 7, 2012, at Mills River. Rhizomes were planted 0.5 m apart within the plots at a depth of 5 cm for a total of nine plants m⁻². To alleviate possible edge effects, a buffer row of plants was planted 0.5 m outside of each plot. Irrigation was used at Mills River to allow establishment of the plants. At both sites, the herbicides 2,4-D (Southern Agricultural Insecticides, Inc., Boone, NC) and Bicep II Magnum (Syngenta Crop Protection, Greensboro, NC) were used for weed control as needed.

After two months, a count of establishment was made and, at Mills River only, extra giant miscanthus seedlings were transplanted to fill in any gaps. In 2013, plants did not receive any additional fertilizer, irrigation, or herbicide and were allowed to grow the full year undisturbed.

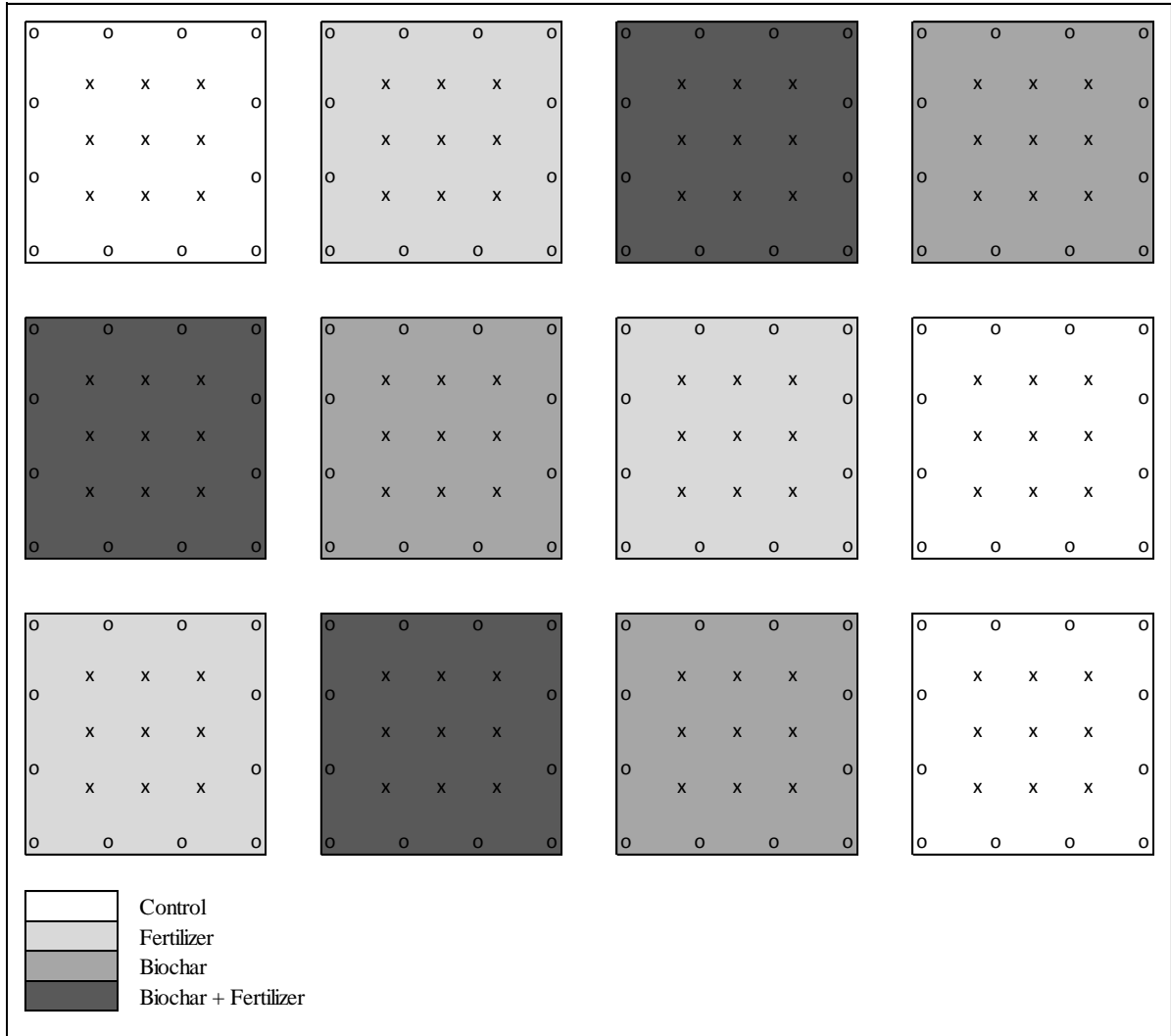


Figure 2. Schematic of a field site. Each “x” represents a single plant that was used in the study, while each “o” represents a buffer plant. Plants within each plot were spaced 0.5 m apart and border plants were 0.5 m outside the plots. The different shadings represent the four treatment combinations. Treatments were blocked by row, but blocks were not statistically significant in the statistical analyses. Each plot was 4 m² in size.

Soil characterization

Soil samples were taken for nutrient (pH, P, K, Ca, Mg, Mn, Zn) and textural analyses at both sites (Agricultural and Environmental Services Laboratories, University of Georgia, Athens, GA) by collecting three randomly located soil cores of 20 cm depth from each of the three control plots to form a composite sample. This procedure was performed twice at each field site for a total of two composite samples per site. Samples were also taken from both control and biochar plots at each site, using the same technique, in order to perform water retention analyses using a pressure plate apparatus (Department of Soil Science, North Carolina State University, Raleigh, NC).

Gas exchange

In mid-September 2012, before leaf senescence had begun, net photosynthesis (A) and stomatal conductance (g_s) were measured on three randomly selected plants per plot using an open-flow gas exchange system (Li-Cor 6400XT, Li-Cor Inc., Lincoln, Nebraska) equipped with an LED light source and 2x3 cm aperture cuvette. Measurements were taken on a middle tiller of each plant at mid-leaf of the newest fully formed leaf. Conditions were set to near ambient conditions (PAR = $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$, $[\text{CO}_2] = 400 \text{ ppm}$, RH = 60-70%, temperature = 25°C), and leaves were allowed to acclimate for a minimum of 2 and a maximum of 5 minutes before measurements were recorded. All measurements were conducted between 1000 and 1400 h. Plant height and the number of tillers per plant were also recorded at this time. No additional measurements were taken in 2013.

Biomass determination

Plants were harvested on December 11, 2012, at Mills River and on December 19, 2012, at Valle Crucis. Individual plants were cut 5 cm above ground using hand trimmers and placed in separate bags. Plants were dried for 72 hours at 90°C before weighing to determine the total aboveground biomass per plant. At Mills River, the extra seedlings that were planted later in the year were not used because these plants had negligible growth for the first year; therefore, to correct for

any missing plants at both sites, the average plant biomass per plot was calculated and used to express yield as estimated total aboveground biomass per plot area (Mg DW ha⁻¹).

In December 2013, a mechanical harvester was used to cut and weigh plants on site at Mills River. For this harvest, the average plant biomass was calculated using both the plot interior and border plants (21 total per plot). At Valle Crucis, plants were again hand-harvested and weighed. Because there were many missing plants at this field site, a linear regression was used to determine whether average plant biomass increased when there were fewer plants growing in a plot. No significant relationship was found. A subsample of buffer plants was harvested at Valle Crucis to determine whether buffer plant biomass was significantly different from that of the interior plants. Again, no significant relationship was found. Therefore there were likely no biases between field sites for the 2013 yield data due to missing plants. Yield was once again expressed as an estimated total aboveground biomass per plot area (Mg DW ha⁻¹) based on average plant biomass per plot.

Statistical analyses

A t-test was used to determine differences in water holding capacity between control and biochar amended soils at each measured pressure. A two-way randomized block ANOVA was used to determine the main effects and interactions between biochar and fertilizer for the following parameters: emergence, gas exchange parameters, height, number of tillers, and aboveground biomass from both years, which were analyzed separately. For all analyses, data were checked for normality and equal variances. Only stomatal conductance required transformation (log) to obtain normality. Differences at $p < 0.05$ are considered significant. Means are reported \pm standard error. All statistical analyses were conducted using SAS v. 9.3 (SAS Institute, Cary, NC).

Results

Site and soil characteristics

Average maximum and minimum monthly temperatures for both 2012 and 2013 were similar at each field site, as was total monthly precipitation (Fig. 3). Increased precipitation was seen in May, June, July, and August of 2013 compared to 2012, the peak months of the growing season. Soil nutrients and textures for both Mills Rivers and Valle Crucis were not significant different from each other, with the exception of increased Zn at Mills River (Table 1). No significant differences in water holding capacity were found at Mills Rivers, but biochar increased the water holding capacity of the soil at all soil matric potentials at Valle Crucis by an average of 12% ($p < 0.0001$; Fig. 4).

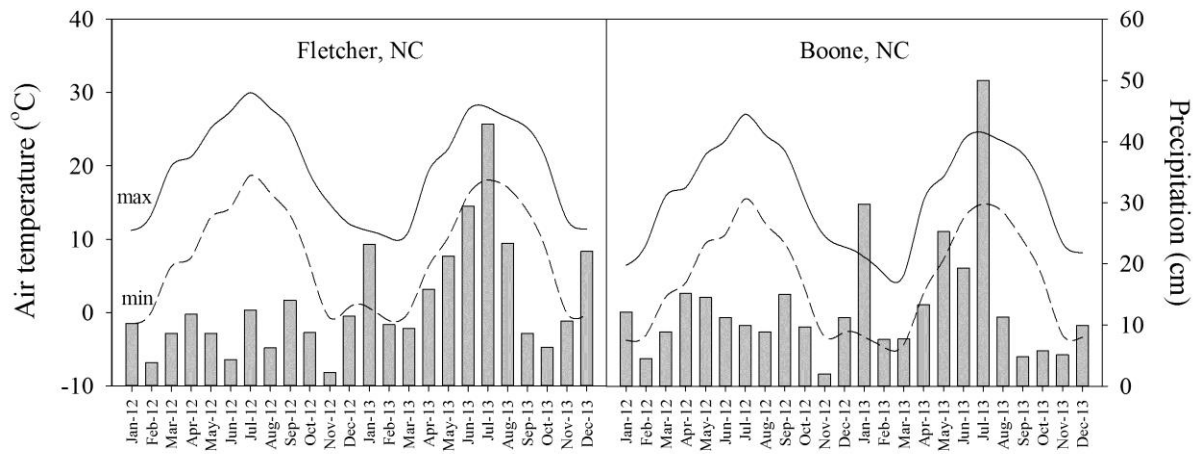


Figure 3. Total monthly precipitation (bars) and average monthly maximum (solid line) and minimum temperatures (dashed line) in 2012 and 2013 from the nearest weather station to each field site.

Table 1. Soil characteristics and nutrient content for Mills River and Valle Crucis. Values for sand, silt clay in percent while all nutrient concentrations are mg kg⁻¹.

Parameters	Mills River	Valle Crucis
Soil Type	Sandy Clay Loam	Clay Loam
Sand	43.8	27.8
Silt	24.0	37.0
Clay	32.2	35.2
pH	5.79	6.67
Ca	809	1347
K	130	176
Mg	185	322
Mn	21	33
P	38	48
Zn	2.7	1.8

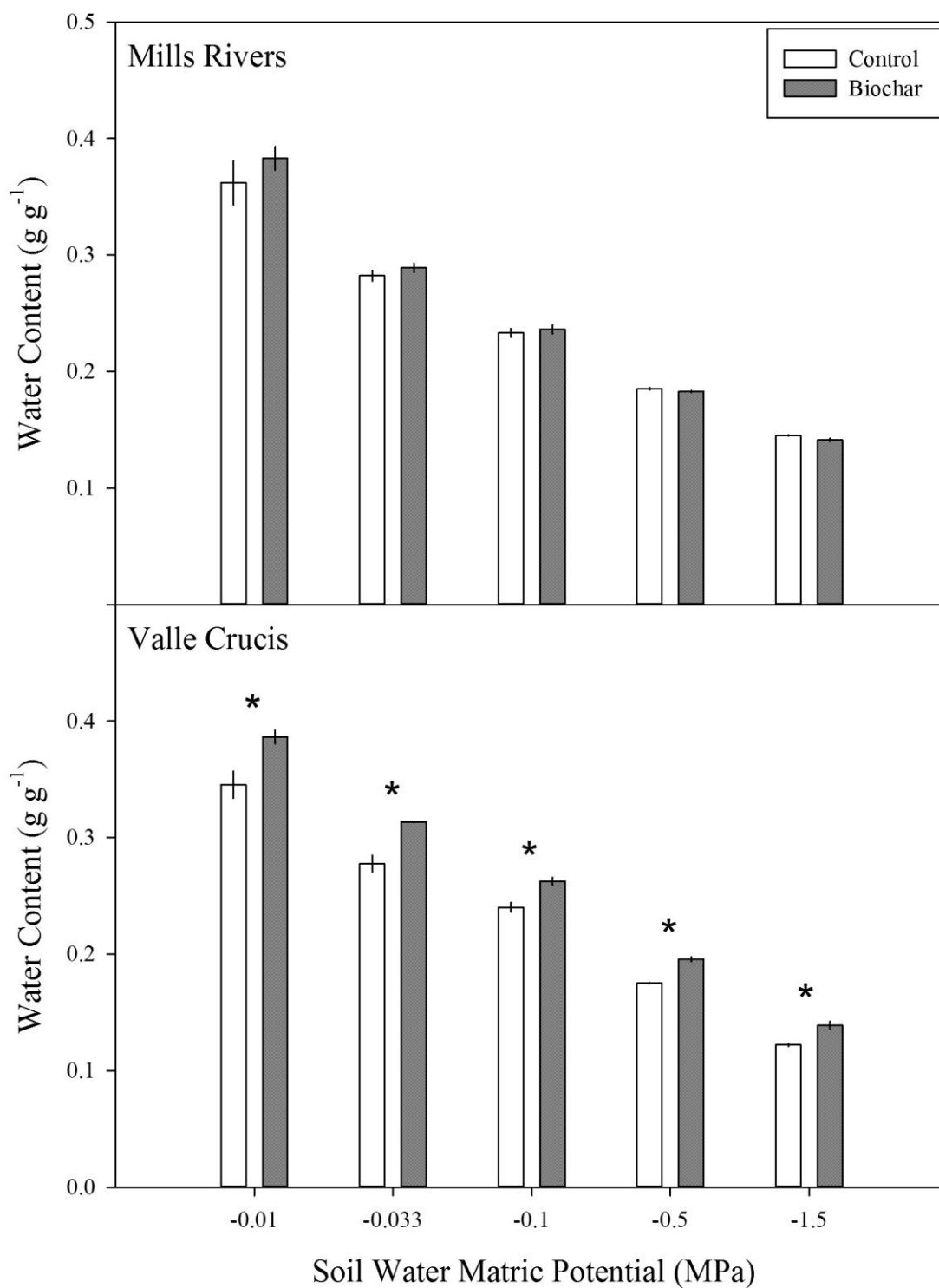


Figure 4. Water holding capacity of soils from (top) Mills Rivers and (bottom) Valle Crucis. Asterisks indicate significant differences ($p < 0.05$) between control and biochar amended soils. Bars are mean \pm se. $n = 3$.

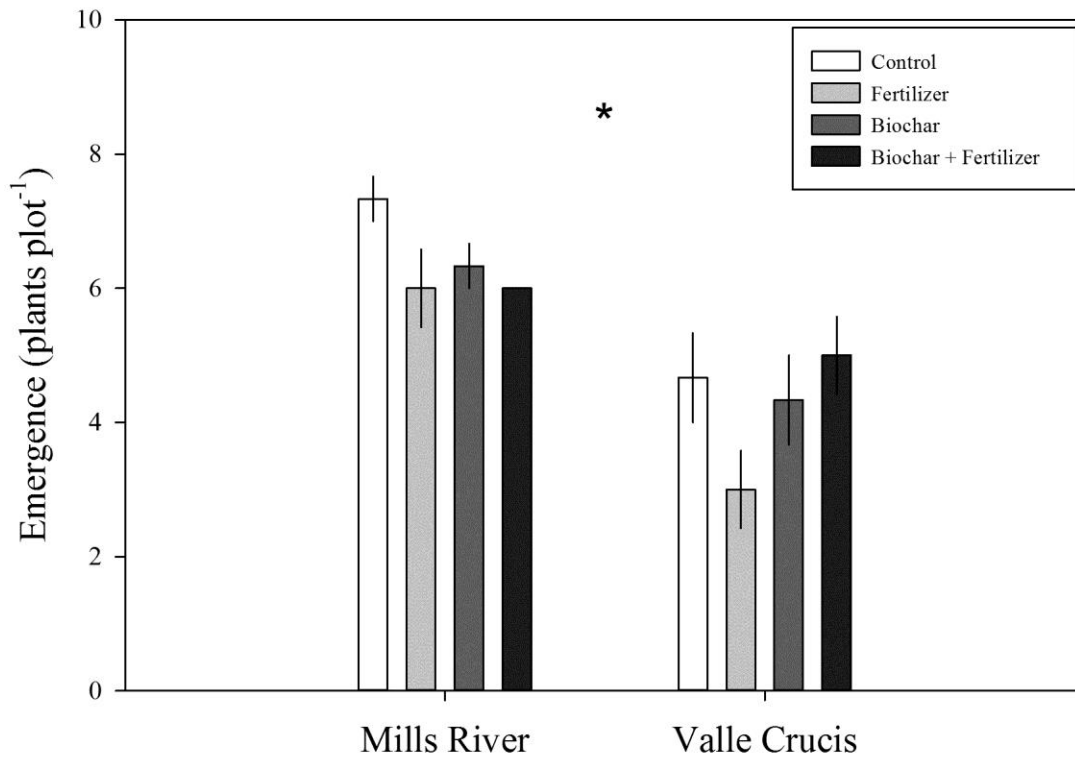


Figure 5. Emergence of giant miscanthus rhizomes per plot out of possible nine. The asterisk indicates a significant difference ($p < 0.05$) between Mills River and Valle Crucis. Bars are mean \pm se. $n = 3$.

Plant properties

For all parameters, blocking was found to be nonsignificant at both field sites. No significant differences in emergence were found among treatment plots within each field site, therefore data were pooled, and a t-test was used to determine significant differences between sites. Emergence was found to be greater ($p < 0.0001$) at Mills Rivers (6.4 ± 0.23 plants plot⁻¹) than at Valle Crucis (4.3 ± 0.35 plants plot⁻¹; Fig. 5). At Valle Crucis, neither biochar ($p = 0.9446$) nor fertilizer ($p = 0.5069$) had an effect on plant height. The same pattern was seen for tillering at Valle Crucis in that neither biochar ($p = 0.9911$) nor fertilizer ($p = 0.1083$) had an effect. At Mills River, however, fertilizer application increased height by 12% ($p = 0.0128$; Fig. 6) and tillering by 44% ($p = 0.0075$; Fig. 7) but biochar did not have an effect on either height ($p = 0.3538$) or tillering ($p = 0.2660$). No significant interactions were found among treatment plots within either field site.

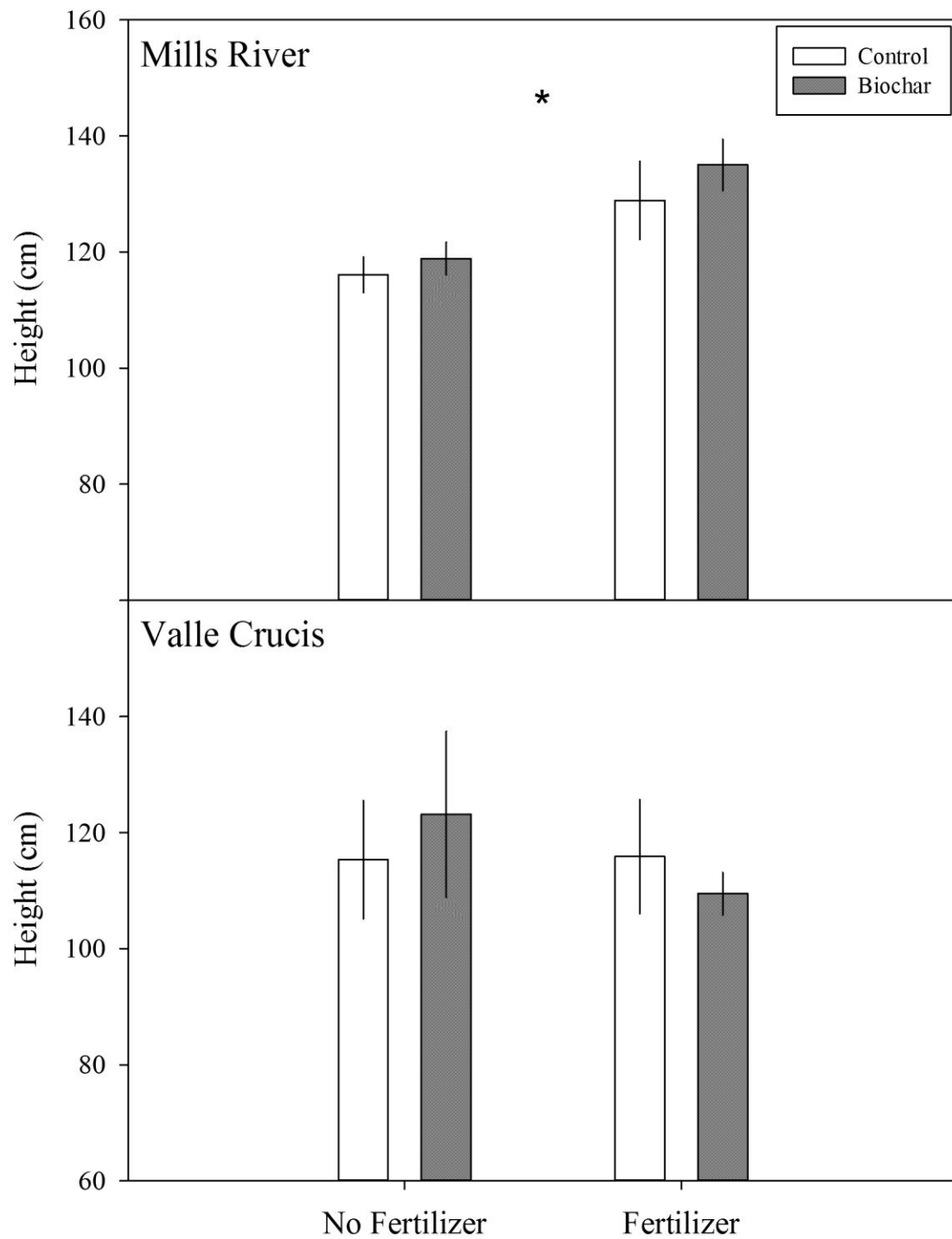


Figure 6. Height of giant miscanthus at (top) Mills River and (bottom) Valle Crucis. The asterisk indicates a significant difference ($p < 0.05$) between fertilized and non-fertilized plants. Bars are mean \pm se. $n = 3$.

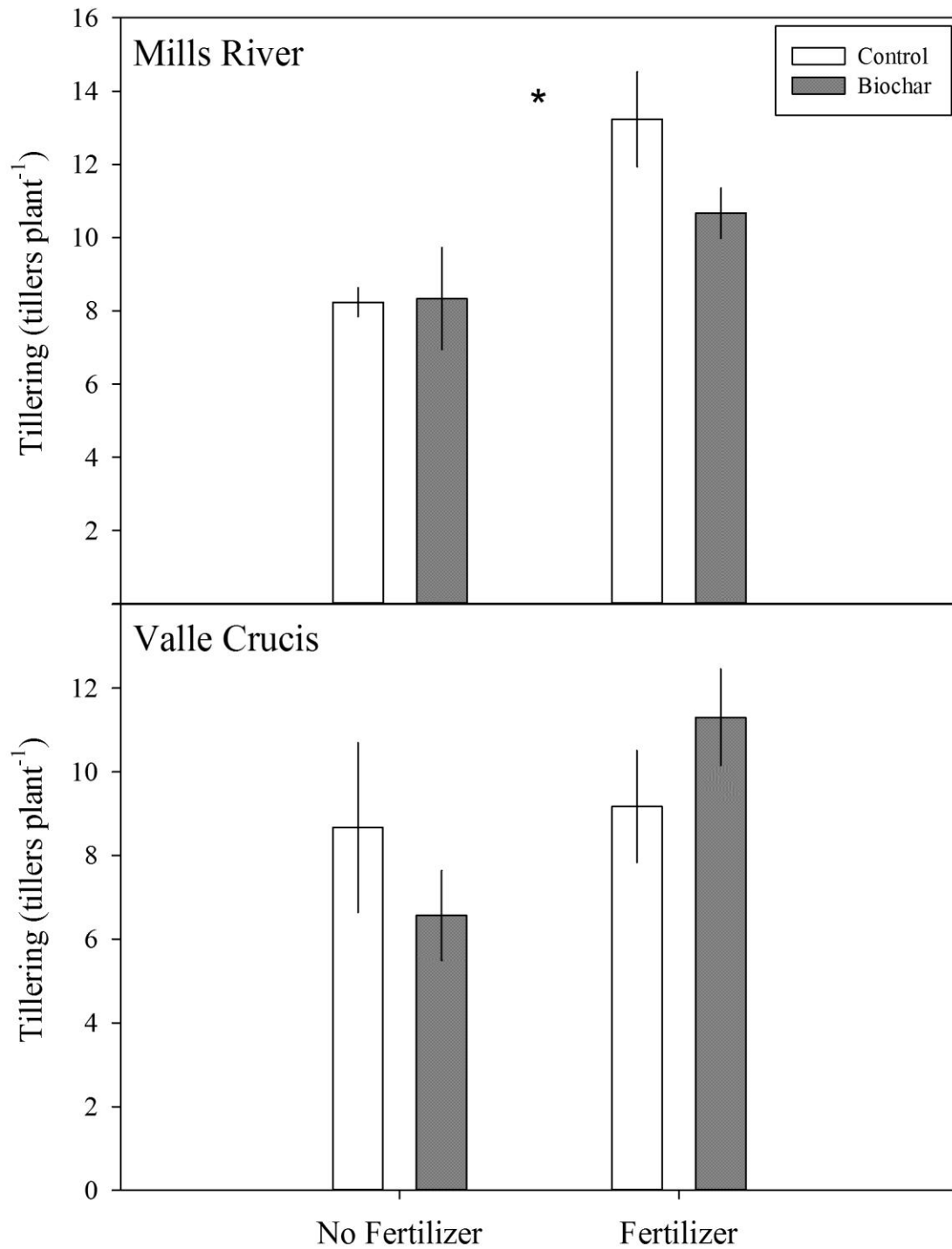


Figure 7. Tillering of giant miscanthus at (top) Mills River and (bottom) Valle Crucis. The asterisk indicates a significant difference ($p < 0.05$) between fertilized and non-fertilized plants. Bars are mean \pm se. $n = 3$.

Gas exchange

Photosynthetic rates, stomatal conductance, and the C_i/C_a ratio were not significantly different among treatment plots within each field site, so data were pooled for each site. Blocking was also found to be nonsignificant for all gas exchange parameters. Photosynthetic rates were greater ($p = 0.0097$) at Mills River ($30.39 \pm 0.736 \mu\text{mol m}^{-2} \text{s}^{-1}$) than at Valle Crucis ($27.24 \pm 0.834 \mu\text{mol m}^{-2} \text{s}^{-1}$; Fig. 8a). Stomatal conductance was also greater ($p = 0.0386$) at Mills River ($0.304 \pm 0.0247 \text{ mol m}^{-2} \text{s}^{-1}$) than at Valle Crucis ($0.246 \pm 0.0083 \text{ mol m}^{-2} \text{s}^{-1}$; Fig. 8b). There was no significant site effect on the C_i/C_a ratio ($p = 0.8882$; Fig. 8c).

Yield and survival

After correcting for the loss of plants in 2012, first year yields were significantly greater ($p = 0.0018$) at Mills River than at Valle Crucis with an average plot yield of $1.65 \pm 0.140 \text{ Mg DW ha}^{-1}$ and $1.00 \pm 0.122 \text{ Mg DW ha}^{-1}$, respectively (Fig. 9). First year overwinter survival rates of rhizomes were 100% for both field sites. Second year yields in 2013 were not significantly different between field sites ($p = 0.2856$), but were 10 times greater than first year yields, with an average plot yield of $15.57 \pm 0.716 \text{ Mg DW ha}^{-1}$ at Mills River and $16.95 \pm 1.033 \text{ Mg DW ha}^{-1}$ at Valle Crucis (Fig. 9). In both years, neither biochar nor fertilizer had a significant effect on yields, nor was there any blocking effect.

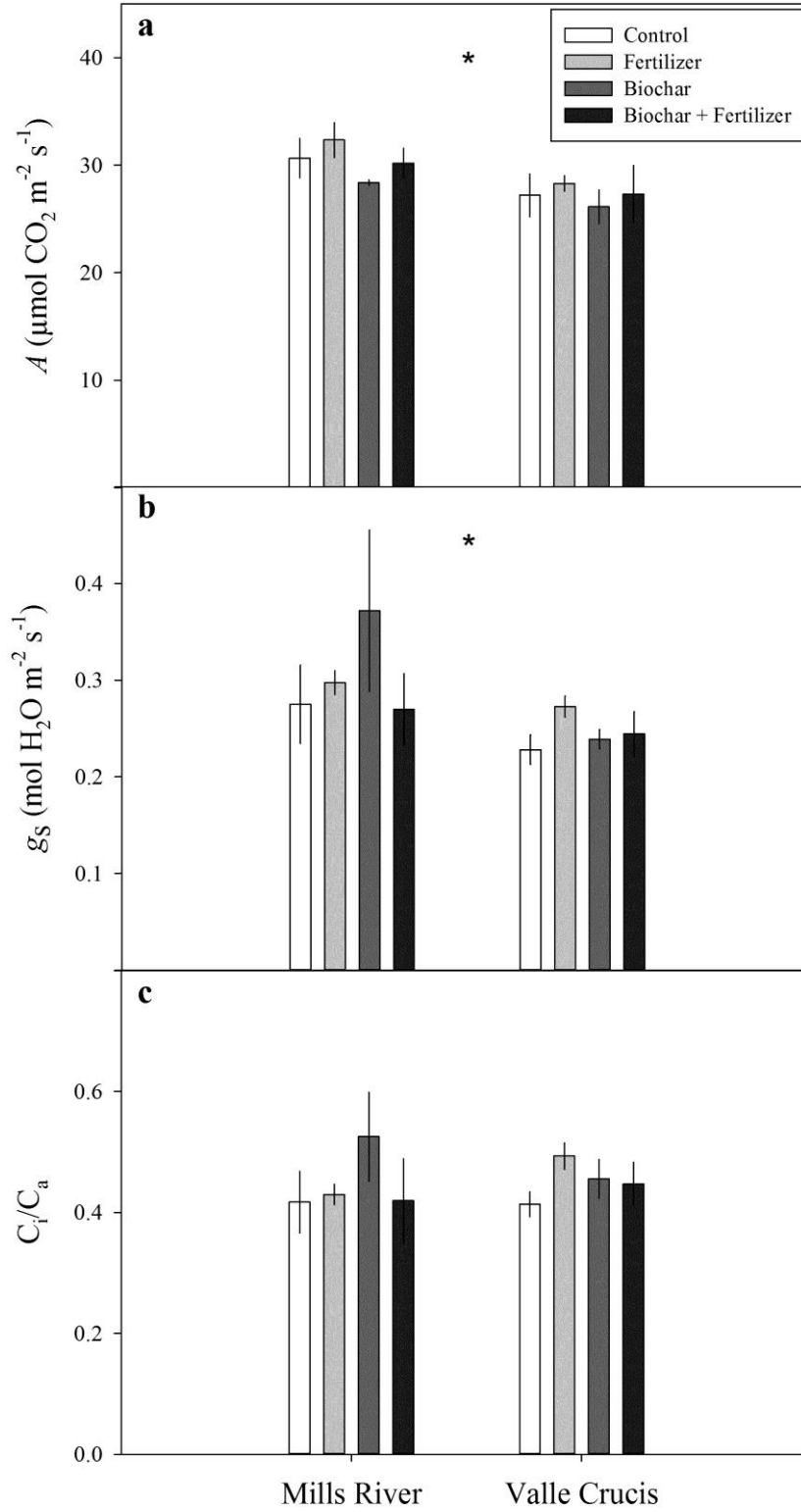


Figure 8. (a) Net photosynthetic rates, (b) stomatal conductances and (c) the C_i/C_a ratios of giant miscanthus at each field site. Asterisks indicate significant differences ($p < 0.05$) between Mills River and Valle Crucis. Bars are mean \pm se. $n = 3$.

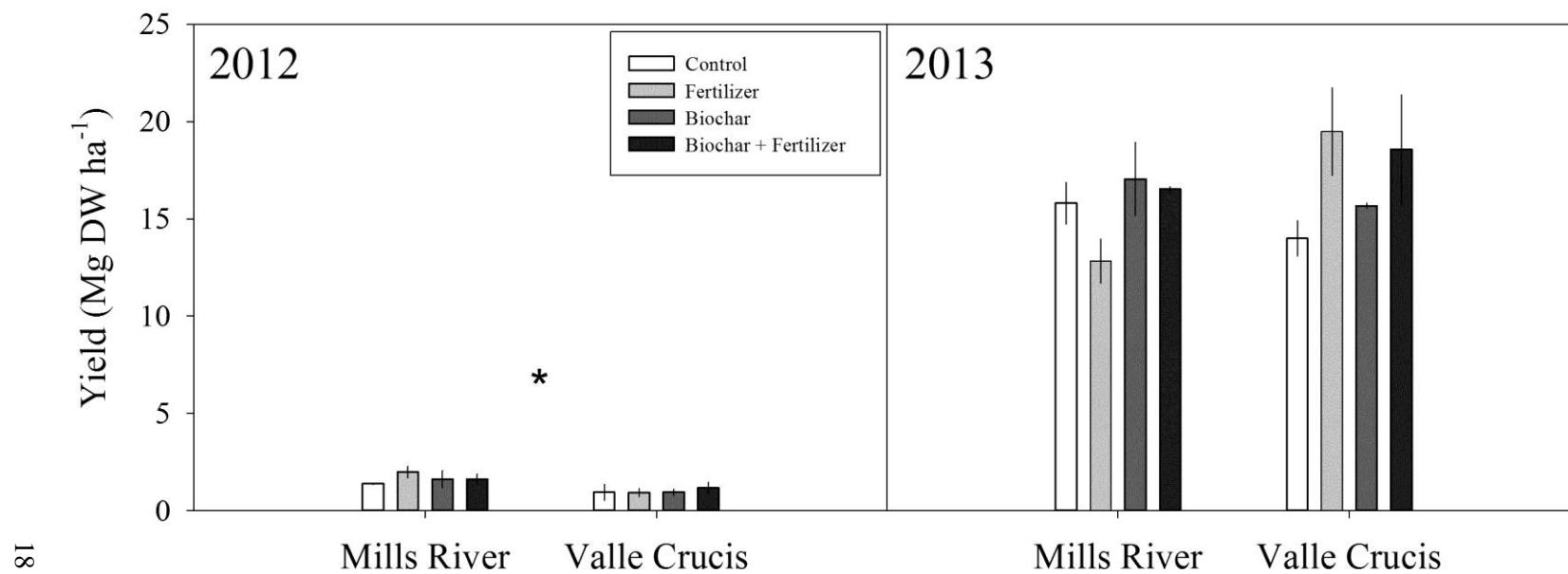


Figure 9. Dry matter yield of giant miscanthus from each field site in 2012 and 2013. The asterisk indicates a significant difference ($p < 0.05$) between Mills River and Valle Crucis in 2012. Bars are mean \pm se. $n = 3$.

Discussion

This experiment demonstrates that giant miscanthus is not only capable of establishment and growth in the soils of the Western North Carolina mountains, but it is also able to produce yields comparable to those of previous studies conducted in geographically different areas. Giant miscanthus has a history of being successfully grown in various regions around Europe, producing yields ranging anywhere from 4 – 44 Mg DW ha⁻¹ depending on the agricultural practices being used (Lewandowski *et al.*, 2000), but cultivation of giant miscanthus in the United States is relatively new, and determining a suitable geographic range is important for its establishment as a sustainable bioenergy crop.

My findings are consistent with those of several other recent studies from the United States that found first year yields of giant miscanthus were relatively low, as the plants established themselves, but second year yields greatly increased (Table 2). Stands of giant miscanthus are also not considered mature until after at least three years of growth (Heaton *et al.*, 2004), so it is reasonable to expect that yields may be even greater in subsequent years. In some cases, second year yields from both of my field sites were greater than reported third year yields in Oklahoma (Kering *et al.*, 2012) and Illinois (Heaton *et al.*, 2008). It should be noted though that total precipitation in summer of 2013 at both Mills River and Valle Crucis was greater than total precipitation in summer of 2012, which likely contributed to increased yield.

I also investigated whether fertilizer might be able to increase the growth of giant miscanthus. Both tillering and plant height were increased with the addition of fertilizer at Mills Rivers during the first year, possibly due to increases in stem elongation, but without adding new biomass, which is why the height increase did not translate to a significant yield improvement. In fact, fertilizer had no effect on yields at both sites in both years in my study. These findings are consistent with recent studies showing no increases in yields with varying rates of fertilizer ranging from 120 kg N ha⁻¹ up to 168 kg N ha⁻¹ (Behnke *et al.*, 2012; Kering *et al.*, 2012; Maughan *et al.*, 2012; Palmer *et al.*, 2014). When fertilizer was applied at a rate of 224 kg N ha⁻¹, though, yields of giant miscanthus increased by

Table 2. Comparative yields of giant miscanthus from studies conducted in the United States. All fertilization rates based on reported values of N from studies. In the current study, fertilizer inputs also include P and K applied at the same rate as N. If multiple yields were presented with different N applications, an average across all N applications was taken. Data from the current study are in bold.

Location	Latitude, Longitude	Elevation (m)	Stand Age (years)	Yield (Mg DW ha ⁻¹)	Notes on Fertilization	Source
Shabbona, IL	41°51'00" N, 88°50'60" W	265	3 - 5	13.7 - 29.9	Fertilized during establishment	Heaton <i>et al.</i> 2008
Mead, NE	41°10'07" N, 96°28'10" W	355	2 3	15.6 27.4	0 – 120 k N ha ⁻¹ yr ⁻¹ , no effect	Maughan <i>et al.</i> 2012
Adelphia, NJ	40°13'31" N, 74°14'54" W	30	2 3	16.9 9.7	0 – 120 k N ha ⁻¹ yr ⁻¹ , no effect	Maughan <i>et al.</i> 2012
Urbana, IL	40°06'20" N, 88°19'18" W	230	1 2	3.1 15.9	0 – 120 k N ha ⁻¹ yr ⁻¹ , no effect	Behnke <i>et al.</i> 2012
Urbana, IL	40°04'48" N, 88°13'48" W	220	3 - 5	25.1 - 44.1	Fertilized during establishment	Heaton <i>et al.</i> 2008
Troy, KS	39°77' N, 95°12' W	335	1 2	4.0 13.7	Fertilized during establishment, 45 kg N ha ⁻¹	Propheter and Staggenborg 2010
Manhattan, KS	39°11' N, 96°35' W	310	1 2	2.7 11.8	Fertilized during establishment, 45 kg N ha ⁻¹	Propheter and Staggenborg 2010
Lexington, KY	38°07'45" N, 84°30'08" W	270	2 3	17.1 19.0	0 – 120 k N ha ⁻¹ yr ⁻¹ , no effect	Maughan <i>et al.</i> 2012
Simpson, IL	37°27'00" N, 88°40'12" W	135	3 - 5	27.3 - 39.2	Fertilized during establishment	Heaton <i>et al.</i> 2008
Valle Crucis, NC	36°11'49" N, 81°47'09" W	830	1 2	1.00 16.95	0 – 100 kg N ha ⁻¹ , no effect	Current study
Mills River, NC	35°25'24" N, 82°33'33" W	650	1 2	1.65 15.57	0 – 100 kg N ha ⁻¹ , no effect	Current study
Mills River, NC	35°25'24" N, 82°33'33" W	650	1 2 3 - 4	5.1 18.2 17.7 - 19.0	0 – 134 kg N ha ⁻¹ yr ⁻¹ , no effect	Palmer <i>et al.</i> 2014
Wallace, NC	34°45'01" N, 78°04'24" W	20	1 2 3 - 4	1.6 11.4 20.3 - 21.3	0 – 134 kg N ha ⁻¹ yr ⁻¹ , no effect	Palmer <i>et al.</i> 2014
Burneyville, OK	33°53' N, 97°16' W	210	2 3	3.4 6.0	0 – 168 kg N ha ⁻¹ yr ⁻¹ , no effect	Kering <i>et al.</i> 2012

40% compared to no fertilization (Wang, D *et al.*, 2012). It also depends on whether fertilizer is applied only once at planting or during each year of growth. For example, Boehmel *et al.* (2008) found that a low rate of 40 kg N ha⁻¹ was sufficient to increase yields significantly compared to no fertilization and that 80 kg N ha⁻¹ could increase yields even more; but in this case, fertilization was done annually, and the yields were presented only after six years of growth.

The varying responses of giant miscanthus to fertilization could be due, in part, to the different soils in which giant miscanthus is grown, and variation in climatic conditions, such as precipitation and temperature. An earlier study of giant miscanthus also found that when ¹⁵N-labelled fertilization was applied, very little was taken up by the plants (Christian *et al.*, 1997). This suggests that the required N for giant miscanthus comes from another source, such as previously stored nutrients in the rhizomes or through the fixation of soil N. Nitrogen-fixing bacteria are now known to be associated with giant miscanthus (Eckert *et al.*, 2001) and may contribute a significant fraction of the N required for growth. Davis *et al.* (2010) also found that the presence of nitrogen-fixing bacteria would account for a large source of unknown N in a model constructed to balance the energy budget of giant miscanthus. The presence of these bacteria would explain the low N input required for giant miscanthus growth even in marginal soils with low nutrient status.

High yields of giant miscanthus may also be due to relatively high rates of photosynthesis that are maintained well into the growing season. Beale *et al.* (1996) found that maximum rates of photosynthesis of giant miscanthus reached $34.2 \pm 2.4 \mu\text{mol m}^{-2} \text{s}^{-1}$ during the summer months in southern England, but dropped significantly to $19.9 \pm 1.8 \mu\text{mol m}^{-2} \text{s}^{-1}$ in the month of October. More recently, Dohleman *et al.* (2009) measured photosynthetic rates in the range of 20-30 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for giant miscanthus grown in Illinois, and as in England these rates also began to decline by late September and early October. In comparison, my measurements were made in mid-September, and average photosynthetic rates were still high at 30 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in Mills River and just under 30 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in Valle Crucis. This suggests that in the Western North Carolina mountains, these plants are able to continue photosynthesizing at high rates late into the growing season, which may be a major

factor contributing to the high yields at both sites. It would be useful to carry out studies of the relative influences of leaf level photosynthetic rates and length of the growing season (i.e., phenology) on growth and yield of giant miscanthus across its habitable range. Based on the results of my study, phenology might prove to be one of the more important factors influencing yields.

An important consideration for giant miscanthus growth is that propagation must be done using rhizomes, due to the sterility of this plant, and this is not only expensive but also time consuming. Therefore, for this crop to be economically practical as a bioenergy crop in Western North Carolina mountains, it is imperative that rhizome survivorship remain high, even under the harsh conditions that can occur in this region of the state. Giant miscanthus rhizomes are capable of withstanding temperatures as low as -3.5°C before 50% losses are seen (Clifton-Brown & Lewandowski, 2000), and significant losses in shoots are not seen until -8°C (Farrell *et al.*, 2006). Increases in rhizome survivorship can be attained by using rhizomes with a larger mass during the initial planting and by leaving portions of crop residue on site when harvesting in order to buffer soils from cold temperatures (Kucharik *et al.*, 2013).

In contrast to previous studies, Heaton *et al.* (2008) found that in Illinois giant miscanthus rhizomes did not suffer any mortality after initial losses during planting, despite air temperatures reaching -8°C and soil temperatures that were well below freezing. These temperatures are comparable to what is typically seen in the Western North Carolina mountains in winter and suggest that winter hardy genotypes of giant miscanthus may exist that could be preferentially used in colder climates. In my experiment, however, average monthly air temperatures during the winters of 2012-2013 did not reach below -5°C , and that could have contributed to their high survival rates. It is important to continue to follow the survival and growth of giant miscanthus in this mountain region for several more years in order to encompass a wider range of minimum temperatures, before making definitive statements about the long-term potential of this crop here.

A second goal of this project was to determine if biochar could increase nutrient and water status of the soil, thereby raising soil quality enough to increase yields. Biochar can increase the

cation exchange capacity of soils (Liang *et al.*, 2006), soil pH (Vaccari *et al.*, 2011), and water holding capacity (Laird *et al.*, 2010), which would improve nutrient and water availability in the soils and thereby improve growing conditions for plants. While biochar appeared to be playing some role in changing soil structure, as evidenced by increases in water holding capacity at the Valle Crucis site, these changes in soil properties did not have any effect on plant physiology or growth of giant miscanthus. Although other studies have found increased above- and below-ground biomass with biochar addition (Kammann *et al.*, 2011; Vaccari *et al.*, 2011) as well as increased plant height and leaf area (Graber *et al.*, 2010), many of these studies used application rates well above those employed here. I chose a lower application rate that would be more realistic in terms of commercial application due to costs and storage, and this low rate likely explains why no significant effects of biochar, positive or negative, were seen. Although there are some studies that suggest biochar can actually depress growth by immobilizing some nutrients (Bargmann *et al.*, 2014), the lack of any growth suppression in my study suggests that a relatively low rate of biochar applied to agricultural fields would most likely not hinder growth. In fact, even if it does not stimulate growth, it may still provide additional benefits that were not measured in the present study, such as increased microbial activity (Graber *et al.*, 2010) and decreased nitrate leaching (Bell & Worrall, 2011).

The economic costs of storing and applying biochar at greater rates could outweigh the benefits of potentially increased yields if those yield increases are not sufficiently greater to justify the added costs. Even if growth can be stimulated by high rates of biochar addition, there could be adverse environmental consequences from such high application rates. For example, Major *et al.* (2010) have suggested that as much as 53% of applied biochar can be lost through wind and seepage, which could have severe environmental consequences for waterways downslope from large fields and also for groundwater supplies if the seepage is deep enough.

Currently, there are few standards for the type of biochar applied to fields. Both the source material and production variability can alter the structure of the biochar itself (Lehmann, 2007), which, in turn, can affect the environmental attributes of the biochar and its impacts on soils and

plants. It is clear that more research needs to be done (Verheijen *et al.*, 2014) to better understand the risks and benefits of biochar application and to take into account the range of possibilities with regard to carbon sequestration, its effects on both soil and plants, and the economic and environmental costs.

As climates continue to change, the need for economical renewable fuel sources will become even more critical. One of the biggest obstacles to overcome will be identifying a crop that can grow with minimal fertilizer and water inputs and which can also be cultivated in marginal soils in a variety of climates, while not competing with land currently used for food crop production. Giant miscanthus has the potential to be a high yielding dedicated bioenergy crop in the Western North Carolina mountains because of its low fertilization or irrigation requirements. The results of this study show that two-year yields are comparable to those in other regions of the country. Biochar did not alter the physiology or yield of giant miscanthus, but at one site, fertilizer did alter biomass allocation by causing plants to become taller without increasing their yield. It may be fruitful to conduct longer term studies with higher biochar and fertilizer application rates, to understand more fully the range of impacts of these factors on the growth, physiology, and yield of giant miscanthus in the Western North Carolina mountains as well as in other high elevation sites in the eastern United States.

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Chapter 2: Physiological responses of *Miscanthus × giganteus* to a short-term drought and rewatering in a biochar-amended soil

Abstract

Miscanthus × giganteus, or giant miscanthus, has emerged as a potential high-yield bioenergy crop due, in part, to its ability to grow in marginal soils; however, water availability is a limiting factor worldwide for bioenergy crop production, particularly on marginal lands with poor quality soils, and prolonged drought conditions can significantly decrease productivity in giant miscanthus. The goals of this study were to understand how giant miscanthus responds physiologically to short-term drought and whether biochar can alleviate drought stress by improving soil water status. Giant miscanthus rhizomes were grown for 75 days in either soil-only or soil amended with biochar at a rate of 50 t ha⁻¹. Drought was induced in half of the plants by withholding watering and physiological measurements (plant water potential, gas exchange, chlorophyll fluorescence) were made every three days throughout the drought period and during a rewatering event. Biochar had no role in alleviating drought stress, and increased soil water status occurred only under well-watered conditions. Although soil volumetric water content began to decrease immediately with lack of watering, most physiological parameters, including leaf water potential, photosynthetic rates and stomatal conductance, did not decrease until water content had reached 38% of well-watered levels, 12 days after drought was induced. After rewatering, leaf water potential recovered to within 90% of pre-drought rates within one day while gas exchange took three days to recover. No differences were seen in total aboveground biomass between plants grown with and without biochar, despite higher soil water status prior to drought, nor was there any effect of drought, probably due to the short duration

of the treatment. The ability of giant miscanthus to recover quickly suggests that short-term drought impacts could be eliminated in a short time, contributing to its ability to grow in marginal soils.

Introduction

There is growing concern over the exponential rise in CO₂ and other greenhouse gases (GHGs) in our atmosphere. If GHG concentrations continue to increase at the current rate, there will be serious consequences due to global climate change including warming, changes in precipitation, rising sea levels, ocean acidification (IPCC, 2007), and changes in species habitat ranges (Root *et al.*, 2003). Atmospheric CO₂ concentrations have risen from 280 ppm in preindustrial times to nearly 400 ppm currently (IPCC, 2007), mainly due to anthropogenic inputs, such as the burning of fossil fuels and land use change (Macías & Arbestain, 2010).

Two primary approaches for mitigating increasing GHG concentrations are currently being explored. The first is the use of alternative fuels, such as solar power, wind power, and bioenergy, in order to reduce the input of new gases that arise from the burning of fossil fuels. The second is the removal of GHGs already present in the atmosphere by means of enhanced carbon sequestration, which can be accomplished by a variety of means, including the planting of forests or improved agricultural techniques that enhance carbon sequestration in the soil.

Of the many candidates being tested as a dedicated bioenergy crop, *Miscanthus × giganteus* Greef et Deu (Greef & Deuter, 1993), hereafter giant miscanthus, has received great interest both in the United Kingdom and the United States due to its capacity to produce high yields under low nutrient conditions (Lewandowski *et al.*, 2000). Because giant miscanthus is a sterile hybrid, there is little risk of invasiveness, unlike one of its parent species, *M. sinensis* (Jørgensen, 2011). Additionally, the C₄ photosynthetic pathway of giant miscanthus allows for increased nutrient and water-use efficiency compared to C₃ plants (Brown, 1978; Sage & Monson, 1999). Recent research suggests giant miscanthus may tolerate colder environments than typical C₄ plants (Heaton *et al.*, 2008), which would contribute to its ability to be grown over a large geographic range.

Even with increased water-use efficiency, giant miscanthus is at risk for yield loss due to limited water availability (Richter *et al.*, 2008; Ings *et al.*, 2013). In some regions, water availability is projected to be more limiting in the future with global climate change (Dai, 2011), and maintaining

relatively high yields under drought is one of the most challenging aspects of producing bioenergy that can compete with conventional fossil fuels (Oliver *et al.*, 2009). Irrigation is often not an option, as it is not economically feasible in the long-term; therefore, either drought tolerant genotypes of giant miscanthus must be identified (Clifton-Brown *et al.*, 2002) or the water status of soils in which giant miscanthus is being grown must be improved.

One possible mechanism to increase soil water status is through the use of biochar soil amendments. Biochar, a black carbon compound produced through pyrolysis of organic matter, has been shown of being capable of altering soil texture, porosity, and density (Atkinson *et al.*, 2010) due to its porous structure, which can lead to an increase in the water holding capacity of soils (Laird *et al.*, 2010; Kammann *et al.*, 2011; Karhu *et al.*, 2011). Additional benefits of biochar soil amendment include increased cation exchange capacity (Liang *et al.*, 2006) and increased soil pH (Vaccari *et al.*, 2011), which can have positive effects on plant growth (Graber *et al.*, 2010) and yield (Kammann *et al.*, 2011; Vaccari *et al.*, 2011). This is especially true for sandy soils and soils of poor quality which will likely be utilized for bioenergy crop production (Quilliam *et al.*, 2012).

It remains unclear, however, whether biochar can be cost effective and beneficial when applied on a larger agricultural scale (Jones *et al.*, 2012). There are also important disadvantages to biochar-amended soils, which may have implications for large-scale field studies. Beesley and Dickinson (2011) reported that the increase in dissolved organic carbon in soils with biochar amendment led to increased copper and lead mobility. Other studies have shown decreased NO₃ availability due to immobilization (Nelson *et al.*, 2011) and decreased plant growth due to decreased N availability (Deenik *et al.*, 2010). Biochar-amended soils also exhibit decreased nitrate leaching, which is generally beneficial, but suggests N immobilization and therefore reduced N available for plant uptake (Bell & Worrall, 2011).

Though biochar has potential advantages for improving soil properties and water status, as well as contributing to increased crop yields, biochar has also emerged as a possible way to increase carbon sequestration in soils and to reduce atmospheric CO₂ by providing a stable, recalcitrant carbon

pool which is highly resistant to decomposition (Woolf *et al.*, 2010). Some estimates suggest that biochar might last up to 1000 years in soils (Vaccari *et al.*, 2011), but the stability of biochar is variable and depends on the conditions used in production (Lehmann, 2007). The porosity of biochar may also provide an ideal habitat for microbes and increase microbial activity in the soil (Solaiman *et al.*, 2010), which may have a positive effect on nutrient cycling and C storage capacity (Atkinson *et al.*, 2010). Nonetheless, few studies exist that examine whether the benefits of biochar could extend to alleviate drought stress in plants, especially for giant miscanthus.

Previous studies documenting the drought responses of giant miscanthus have shown predictable responses, including decreased photosynthetic capacity (Weng, 1993), leaf senescence (Clifton-Brown *et al.*, 2002), decreased leaf area (Clifton-Brown & Lewandowski, 2000), and decreased chlorophyll fluorescence (Ings *et al.*, 2013), but no studies to date have documented the recovery from drought by giant miscanthus for these physiological parameters. Knowing how giant miscanthus will recover from drought is important because if recovery is quick, the deleterious effects of drought would be minimized compared to more sensitive species which may suffer prolonged after-effects from water stress.

The goals of my study were 1) to understand the physiological responses of giant miscanthus to a short-term drought scenario, 2) to determine how quickly giant miscanthus is able to recover its photosynthetic capacity after drought and whether any drought symptoms seen cause noticeable losses in yield, and 3) to determine whether biochar soil amendment could alleviate drought stress of giant miscanthus by improving soil water status and therefore enhance crop yields under drought conditions.

Methods

Experimental setup

Two treatments, water status and biochar addition, were employed on 12 replicate plants per treatment combination. Mortality in some pots reduced the final number of plants per treatment combination to: 8 control (soil-only, well-watered), 10 biochar (biochar-amended, well-watered), 7 drought (soil-only, droughted), and 11 biochar and drought (biochar-amended, droughted). Each pot (inner diameter = 20 cm, height = 30 cm) was filled with topsoil obtained from Appalachian State University property and then sifted to remove any large particles or rocks. Biochar at a rate of 50 t ha⁻¹ with a 30 cm depth (236 g biochar) was applied to half of the pots and thoroughly mixed by hand. The biochar used was obtained from Range Fuels (Soperton, GA) and was produced from pine chips. The production temperature for this biochar is unknown. I chose an average application rate, based on previous studies, for which an increase in plant biomass could be detected (Jeffery *et al.*, 2011; Liu *et al.*, 2013).

Genetically identical rhizomes of giant miscanthus were obtained from Repreve Renewables (Soperton, GA) for use in this experiment. Initially two rhizomes (average mass 21 g) were planted at a depth of 5 cm per pot on May 28, 2013, but extra rhizomes were weeded after sprouting to ensure each pot contained only one plant. All pots received fertilizer one week after sprouting corresponding to rates of 100 kg ha⁻¹ of N, P, and K using Scotts Osmocote 14-14-14 (The Scotts Company, Marysville, OH), a slow release fertilizer. All plants received the same watering regime and were allowed to grow for approximately 75 days.

Soil characterization

Soil samples from a similar source were collected for both nutrient (pH, P, K, Ca, Mg, Mn, Zn) and textural analysis (Agricultural and Environmental Services Laboratories, University of Georgia, Athens, GA). Bulk density of control and biochar-amended soils was determined using pots in which no plants were grown. Samples were also taken from both soil-only and biochar pots to

determine water retention characteristics using a pressure plate apparatus (Department of Soil Science, North Carolina State University, Raleigh, NC). Moisture release curves were then generated in order to transform soil volumetric water content data to soil water potential.

Continuous measurements

After 75 days of growth, drought was induced on designated pots by withholding watering. The drought period lasted 18 days, starting on Julian Day 220 (August 8) after making initial physiological and soil measurements. Measurements were made every three days during the experiment. On Julian Day 238 (August 26), once leaf rolling could be seen and leaf water potentials significantly dropped, all droughted plants were subjected to rewatering and a regular watering regime was resumed for 6 days before final measurements were made and plants were harvested on Julian Day 244 (September 1). All physiological and soil measurements were conducted between 1000 and 1400 h, with the exception of leaf water potential which was measured pre-dawn between 0600 and 0700 h.

Soil volumetric water content (VWC) of each pot was determined using a handheld sensor equipped with 20 cm probes (Hydrosense II, Campbell Scientific, Logan, UT). Measurements were taken at the middle of each pot, avoiding rhizomes.

Predawn leaf water potential (Ψ_{leaf}) was measured on four randomly selected plants per treatment combination using a Scholander pressure chamber (Scholander *et al.*, 1965). A terminal section of a lower leaf, approximately 6 cm long, was used. The same leaf was used for the duration of the experiment, but new sections were cut each time. To eliminate bias, a similar size of leaf material was removed from those plants not being measured each time.

Net photosynthesis (A) and stomatal conductance (g_s) were measured on six randomly selected plants per treatment combination using an open-flow gas exchange system (Li-Cor 6400XT, Li-Cor Inc., Lincoln, Nebraska) equipped with an LED light source and 2x3 cm aperture cuvette. Gas exchange was measured at saturating light ($\text{PAR} = 2000 \mu\text{mol m}^{-2} \text{s}^{-1}$) at ambient conditions ($[\text{CO}_2] =$

400 ppm, RH = 50-60%, temperature = 25°C) on the middle of newest fully formed leaf, and the same leaf was used for the duration of the experiment. Leaves were allowed to acclimate for a minimum of 2 and a maximum of 5 minutes before measurements were recorded.

Chlorophyll fluorescence (F_v/F_m), a measure of the maximum quantum efficiency of photosystem II, was measured on the same plants as gas exchange using a portable fluorometer (Handy PEA, Hansatech Instruments, UK). All plants were dark adapted for at least 30 minutes before being exposed to 1s of a maximum light intensity of $3500 \mu\text{mol m}^{-2} \text{s}^{-1}$.

Final measurements and harvest

Chlorophyll and carotenoid content were measured on the same plants used for gas exchange measurements. Three hole-punches with an area of 0.28 cm^2 each were collected from the middle of the leaf, avoiding the midrib, placed into 3 mL of N,N'-dimethylformamide, and allowed to extract in the dark for 24 hours. Following extraction, absorbances were measured at 470, 647, 664 nm using a UV-VIS spectrophotometer (UV-1201, Shimadzu Corp, Kyoto, Japan). Chlorophyll and carotenoid content (mg/mL) were determined using the equations in Porra (2002).

Specific leaf weight, the ratio of leaf weight to area, of each plant was determined by collecting three hole-punches on the newest fully formed leaf for each plant, drying for 48 hours at 90°C and then weighing. Carbon and nitrogen content were determined from a subsample of leaf material which was dried and ground into a fine powder using a ball mill, then analyzed using a CNS elemental analyzer (FlashEA 1112 Series, Thermo Scientific). The number of tillers per pot was counted before harvesting, and the weight all of the aboveground biomass was measured after drying for 48 hours at 90°C.

Statistical analyses

A t-test was used to determine differences in water holding capacity between control and biochar-amended soils at each individual pressure, and also for bulk density. A two-way repeated

measures ANOVA was used to determine whether biochar or water stress affected soil volumetric water content over time. For leaf water potential, net photosynthesis, stomatal conductance, the C_i/C_a ratio, and chlorophyll fluorescence, no significant biochar effect was found therefore data were pooled into two groups, well-watered and droughted, and a one-way repeated measures ANOVA was used. For all parameters measured over time, the data were split to represent the drought period (Day 220 – Day 238) and the recovery period (Day 239 – Day 244). Moisture release curves were generated using SigmaPlot v. 12.5 (Systat Software Inc., Point Richmond, CA) to transform soil volumetric water content data into soil water potential for both control and biochar-amended soils. A two-way ANOVA was used to determine the main effects and interactions between biochar and water stress for aboveground biomass, chlorophyll and carotenoid content, specific leaf weight, and leaf C:N ratio. For all analyses, differences at $p < 0.05$ are reported as significant. Means are reported as \pm standard error. All statistical analyses were conducted using SAS v. 9.3 (SAS Institute, Cary, NC).

Results

Soil characteristics

The soil used in this experiment had equal proportions of sand and silt and could be best described as a loam (Table 1). Additionally, there appeared to be no obvious deficiency or overabundance of any constituent measured. Biochar significantly lowered ($p = 0.0381$) the bulk density of the soil by 10% (Fig. 1). Biochar also increased the water holding capacity of the soil by ~6% at -0.01 MPa ($p = 0.0271$) and by ~5% at -0.03 MPa ($p = 0.0102$), but at lower potentials no significant differences were observed (Fig. 2). The moisture release curves also showed that at lower potentials control and biochar-amended soils were almost identical (Fig. 3).

Drought responses

Before the drought period began, biochar-amended soils had a greater ($p = 0.0001$) volumetric water content (VWC) than did control soils, which persisted throughout the experiment (Fig. 4). Volumetric water content began to decrease ($p = 0.0006$) 3 days after drought was induced for both soil-only and biochar-amended pots, and continued to decrease throughout the drought period. After 9 days the effects of biochar were no longer apparent when compared to soil-only under drought ($p = 0.0716$).

Leaf water potentials began to decline ($p = 0.0014$) 12 days after drought was induced (Fig. 5). On Day 238, 18 days after drought was induced, leaf water potential of biochar-amended pots had reached -1.96 ± 0.245 MPa compared to -1.45 ± 0.296 MPa for soil-only pots; however, no significant difference ($p = 0.2017$) existed between the two.

Droughted plants began showing declines in photosynthetic rates ($p = 0.0228$), stomatal conductance ($p = 0.0003$), and the C_i/C_a ratio ($p = 0.0001$) 12 days after drought was induced, and these parameters steadily declined for the remainder of the drought period (Fig. 6). By the end of the drought period, photosynthetic rates of droughted plants were, on average, 45% of well-watered plants, reaching as low as $10.7 \pm 1.48 \mu\text{mol m}^{-2} \text{s}^{-1}$ in the biochar-amended pots (Fig. 6a). Stomatal

Table 1. Soil characteristics and nutrient content of the control soil used. Values for sand, silt clay in percent while all nutrient concentrations are mg kg⁻¹.

Parameters	Values
Soil Type	Loam
Sand	40.8
Silt	36.0
Clay	23.2
pH	6.15
Ca	1292
K	173
Mg	252
Mn	43
P	124
Zn	4.6

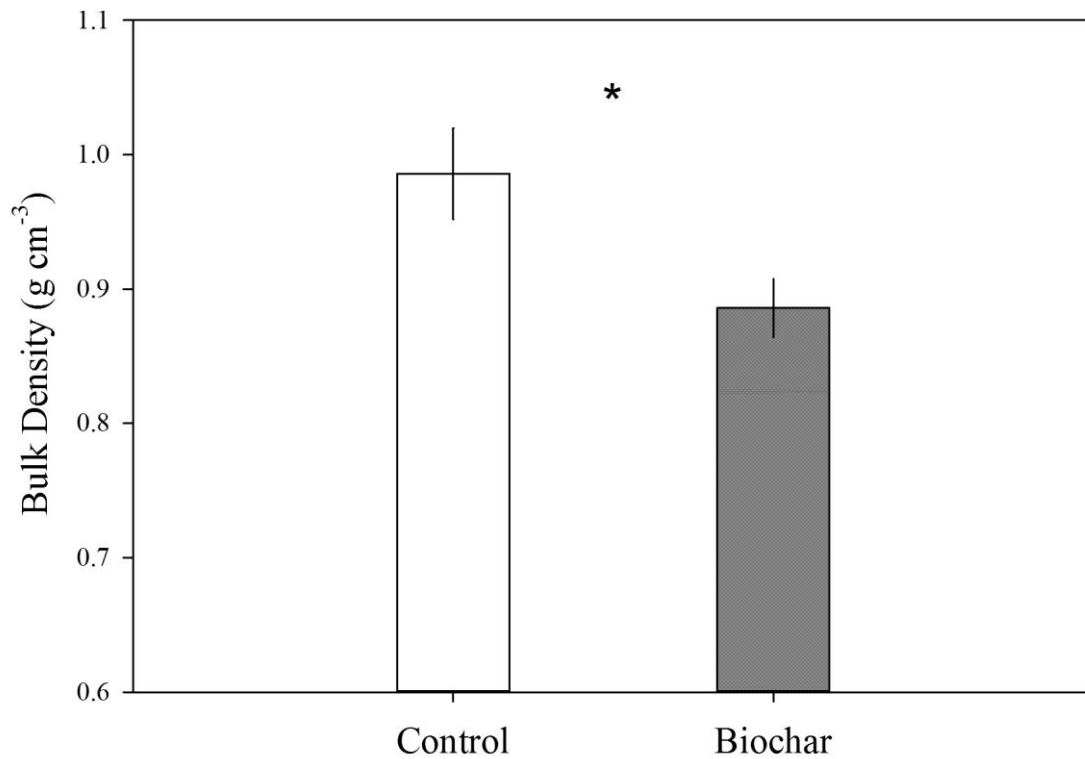


Figure 1. Bulk density (g cm⁻³) of control and biochar-amended soils not subjected to planting or watering. The asterisk indicates a significant difference ($p < 0.05$) between control and biochar-amended soils. Bars are mean \pm se. n = 5.

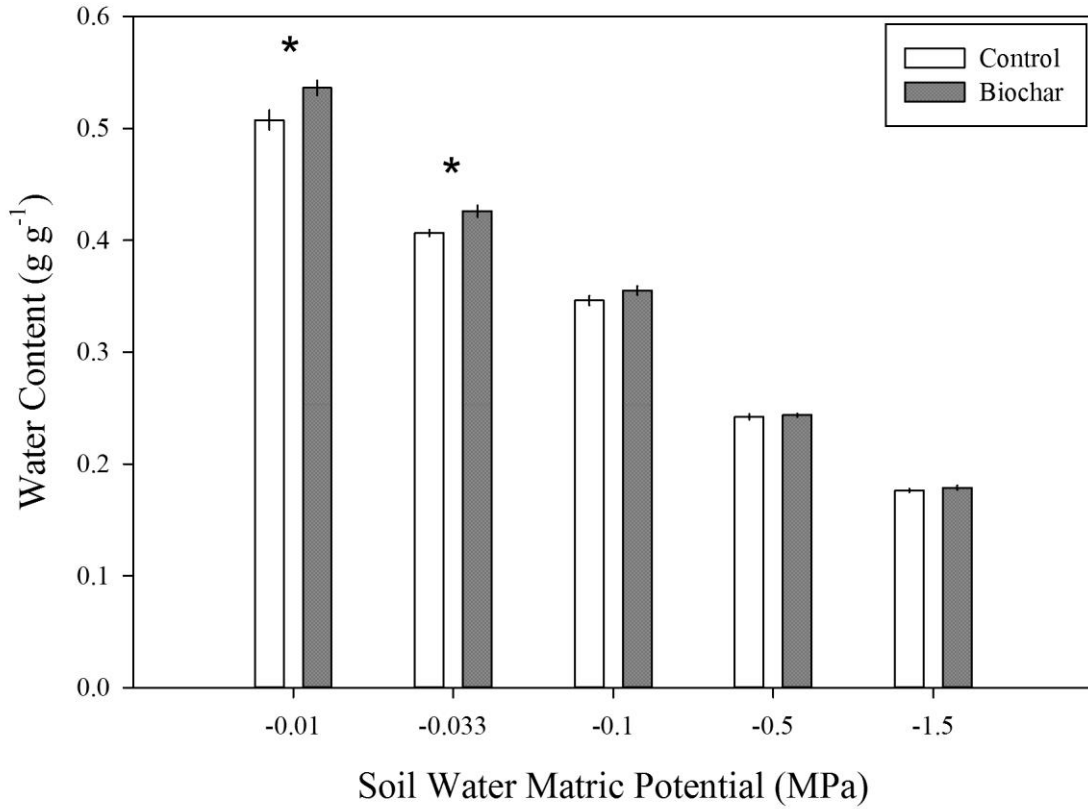


Figure 2. Water holding capacity of control and biochar-amended soils. Asterisks indicate significant differences ($p < 0.05$) between control and biochar-amended soils at a given soil matric potential. Bars are mean \pm se. $n = 3$.

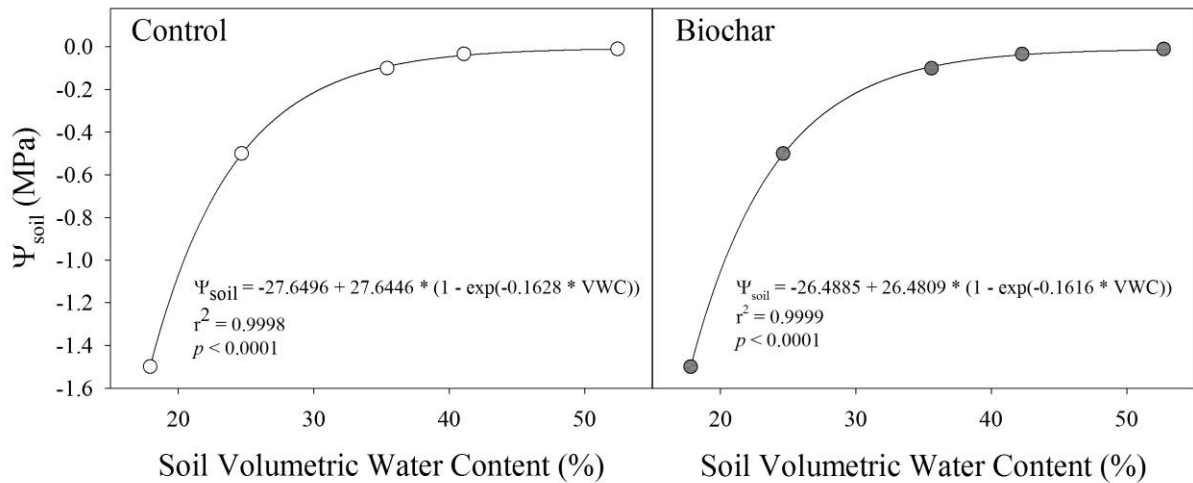


Figure 3. Soil moisture release curves of control and biochar-amended soils. Circles are mean \pm se, though error bars are too small to be seen. $n = 3$.

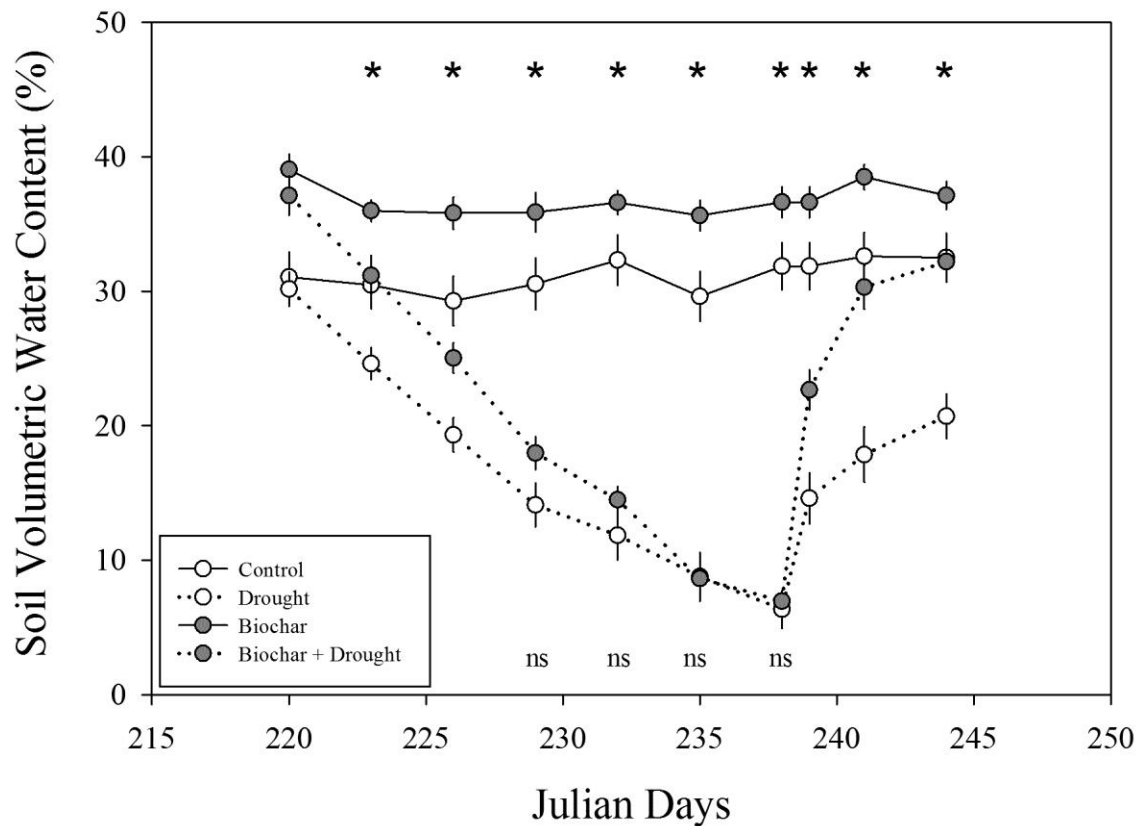


Figure 4. Soil volumetric water content during the drought period (Day 220-238) and rewatering (Day 239-244). For all days, well-watered biochar-amended pots were significantly greater ($p < 0.05$) than well-watered soil-only pots. Asterisks indicate significant differences between well-watered and droughted pots. Days marked “ns” indicated when droughted biochar-amended pots were no longer significantly greater than droughted soil-only pots. Circles are mean \pm se. Sample sizes ranged from 7 to 11.

conductance of droughted plants was 31% lower than well-watered plants (Fig. 6b), while the C_i/C_a ratio was 64% that of well-watered plants (Fig. 6c).

Chlorophyll fluorescence (F_v/F_m) fluctuated throughout the experiment, although well-watered plants had an average F_v/F_m of 0.78 ± 0.003 (Fig. 7). During the drought period, there was no effect due to biochar ($p = 0.7710$), but there was a decline in F_v/F_m of droughted plants on Day 229 ($p = 0.0045$). There also appeared to be a lag in drought response as one day after rewatering, or 19 days after the drought began, the F_v/F_m of droughted plants was lower ($p = 0.0049$) than that of well-watered plants, reaching as low as 0.757 ± 0.003 .

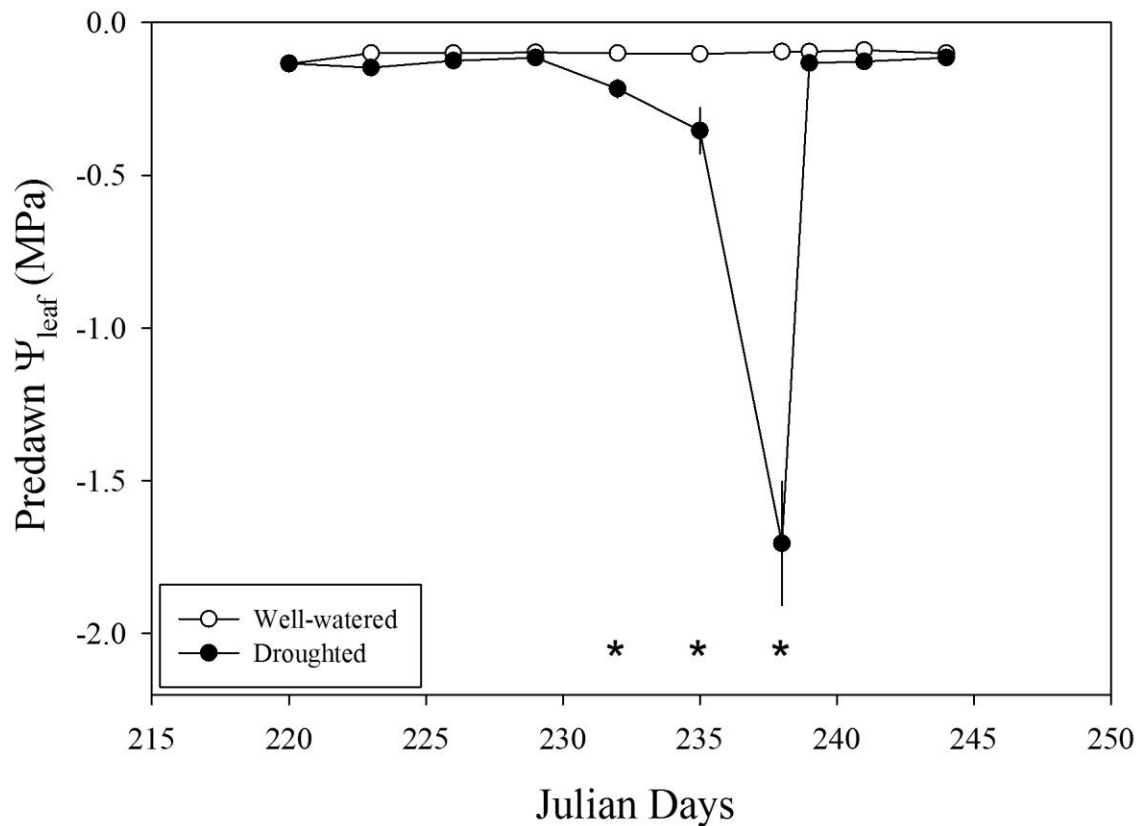


Figure 5. Predawn leaf water potential of giant miscanthus during the drought period (Day 220-238) and rewatering (Day 239-244). Asterisks indicate significant differences ($p < 0.05$) between well-watered and droughted pots. Circles are mean \pm se. $n = 4$.

In addition to the physiological responses measured, leaf rolling of all green leaves in droughted plants began 15 days after drought was induced. The bottom two or three leaves of droughted plants also senesced but did not detach from the stem. The failure of leaves to detach from the stem upon senescence is common for giant miscanthus.

Recovery

One day after rewatering, the VWC of biochar-amended droughted pots had increased ($p = 0.0040$) more so than the soil-only droughted pots (Fig. 4). After one week of resuming a regular watering regime, VWC of both soil-only and biochar-amended pots was still significantly lower ($p < 0.0001$) than the well-watered pots, but VWC of droughted biochar pots recovered to 86% of

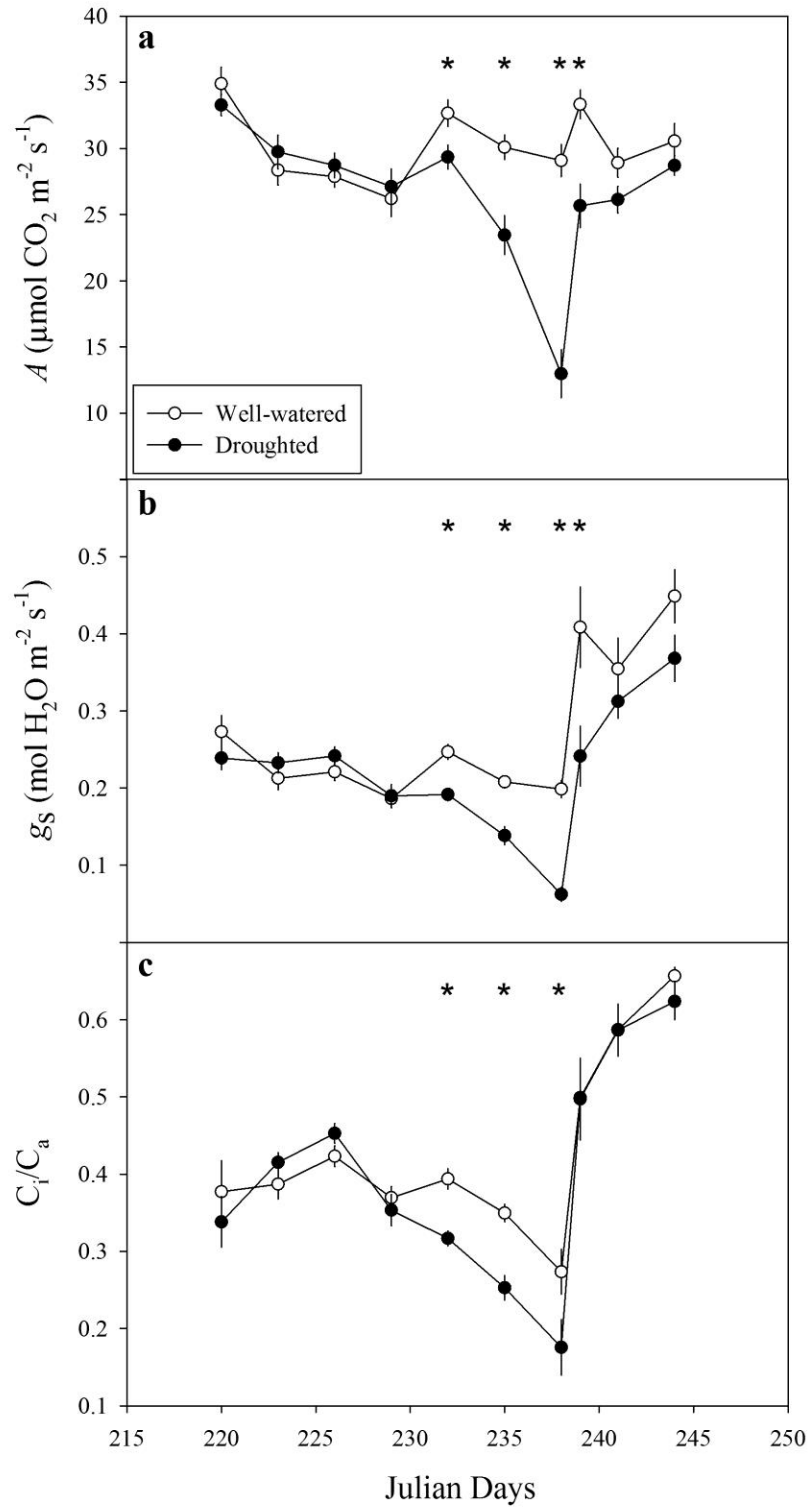


Figure 6. (a) Net photosynthetic rates, (b) stomatal conductance and (c) the C_i/C_a ratio of giant miscanthus during the drought period (Day 220-238) and rewatering (Day 239-244). Asterisks indicate significant differences ($p < 0.05$) between well-watered and droughted pots. Circles are mean \pm se. $n = 6$.

well-watered biochar pots and had reached levels similar to that of the control pots while VWC of soil-only droughted pots only reached 64% of the control pots within the week.

Leaf water potential of droughted plants in both soil-only and biochar-amended pots recovered to well-watered levels within a single day of watering (Fig. 5). Both photosynthetic rates and stomatal conductance of droughted plants were able to recover to 90% of well-watered plant levels within 3 days of rewatering (Fig. 6). The C_i/C_a ratio of the droughted plants was able to recover to 90% of well-watered plant levels within one day of rewatering (Fig. 6c). Although there was a lag in drought response, chlorophyll fluorescence was able to recover to well-watered levels two days after the initial decline was seen, or three days after rewatering (Fig. 7).

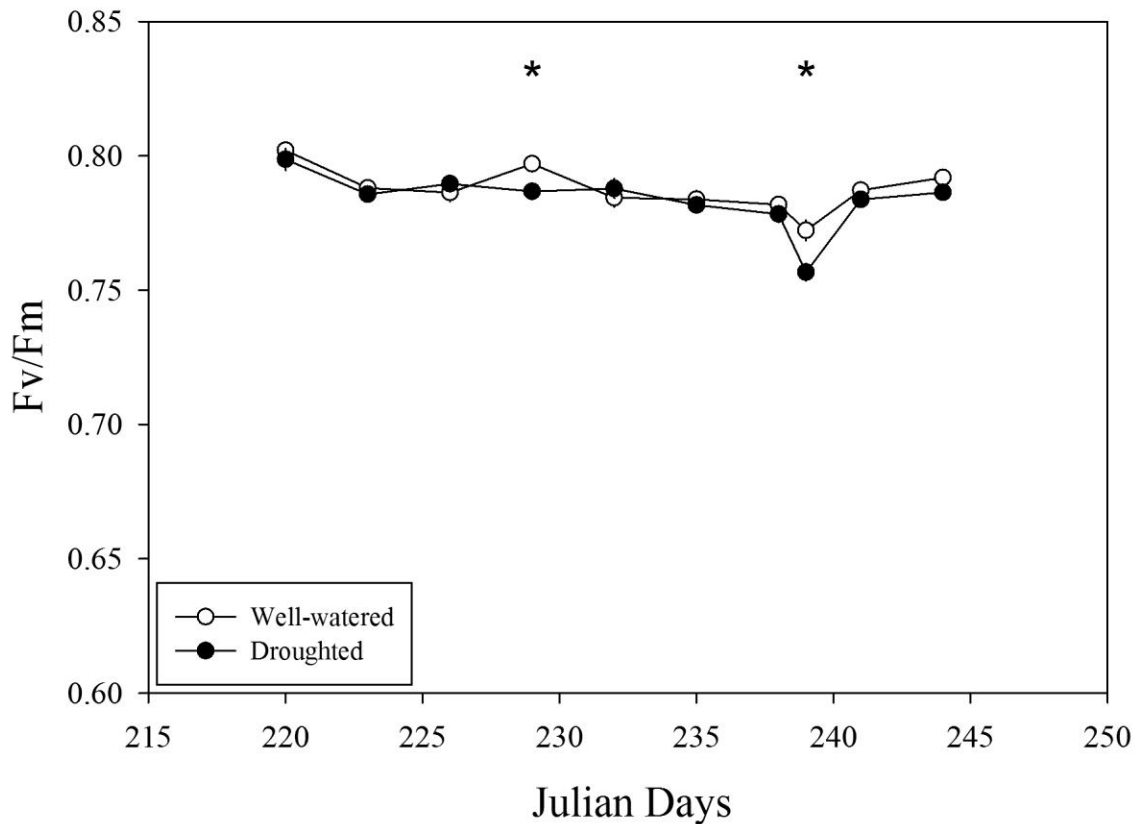


Figure 7. Chlorophyll fluorescence of giant miscanthus during the drought period (Day 220-238) and rewatering (Day 239-244). Asterisks indicate significant differences ($p < 0.05$) between well-watered and droughted pots. Circles are mean \pm se. $n = 6$.

Final measurements and harvest

No differences between biochar treatments ($p = 0.1588$) or water status ($p = 0.6672$) were found for chlorophyll *a*, chlorophyll *b*, chlorophyll *a/b* ratio, and total chlorophyll content (Fig. 8a). Additionally, no differences in carotenoid content of leaves were found either between biochar treatments ($p = 0.0734$) or water status ($p = 0.9236$; Fig. 8b).

Specific leaf weight was not different between biochar ($p = 0.9923$) or water status ($p = 0.1526$) treatments, although there was a trend for greater specific leaf weight in the droughted plants (Fig. 9). Leaf C:N ratios were also not different between biochar ($p = 0.7423$) and water status ($p = 0.2298$; Fig. 10) treatments. Average percent carbon for the leaves across all treatments was $40.8 \pm 0.18\%$, while average percent nitrogen was $1.6 \pm 0.04\%$, leading to an average C:N ratio of 26.4 ± 0.57 .

No differences between biochar treatments ($p = 0.6944$) and water status ($p = 0.9490$) were found for the aboveground biomass per plant, although the droughted plants in soil-only had the lowest average biomass of 25.3 ± 2.75 g (Fig. 11). Average biomass of all other treatment combinations ranged from 27.1 ± 3.38 g to 28.2 ± 1.43 g.

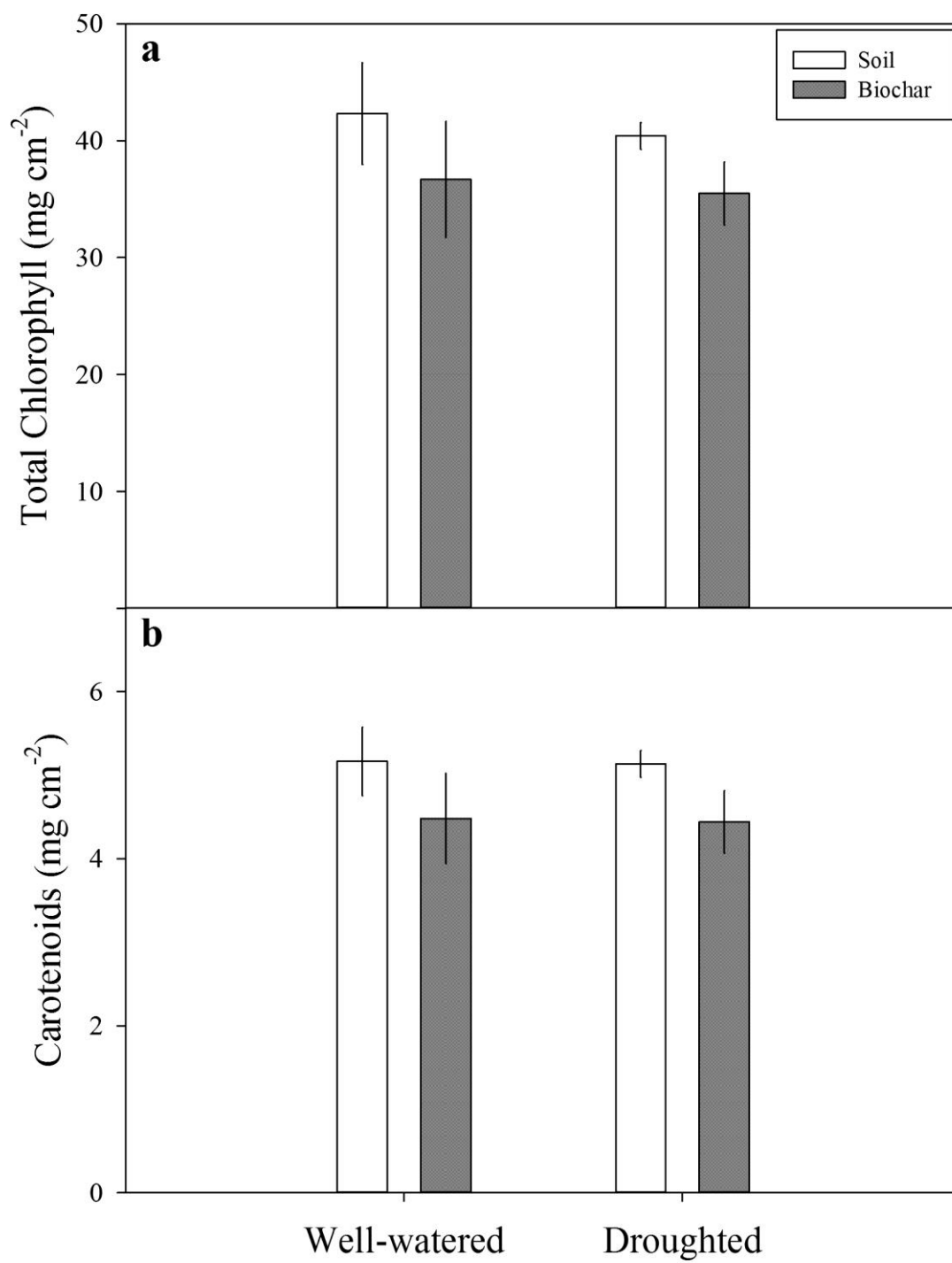


Figure 8. (a) Total chlorophyll and (b) carotenoid content of giant miscanthus leaves at time of harvest. Bars are mean \pm se. n = 6.

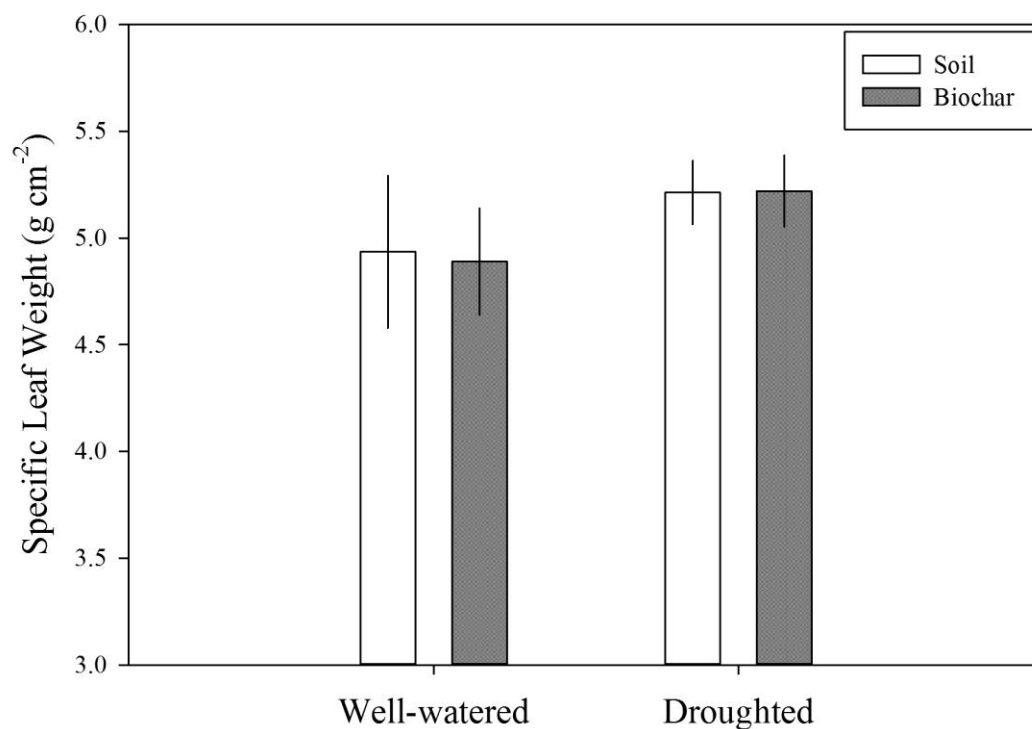


Figure 9. Specific leaf weight of giant miscanthus leaves at time of harvest. Bars are mean \pm se. Sample sizes ranged from 7 to 11.

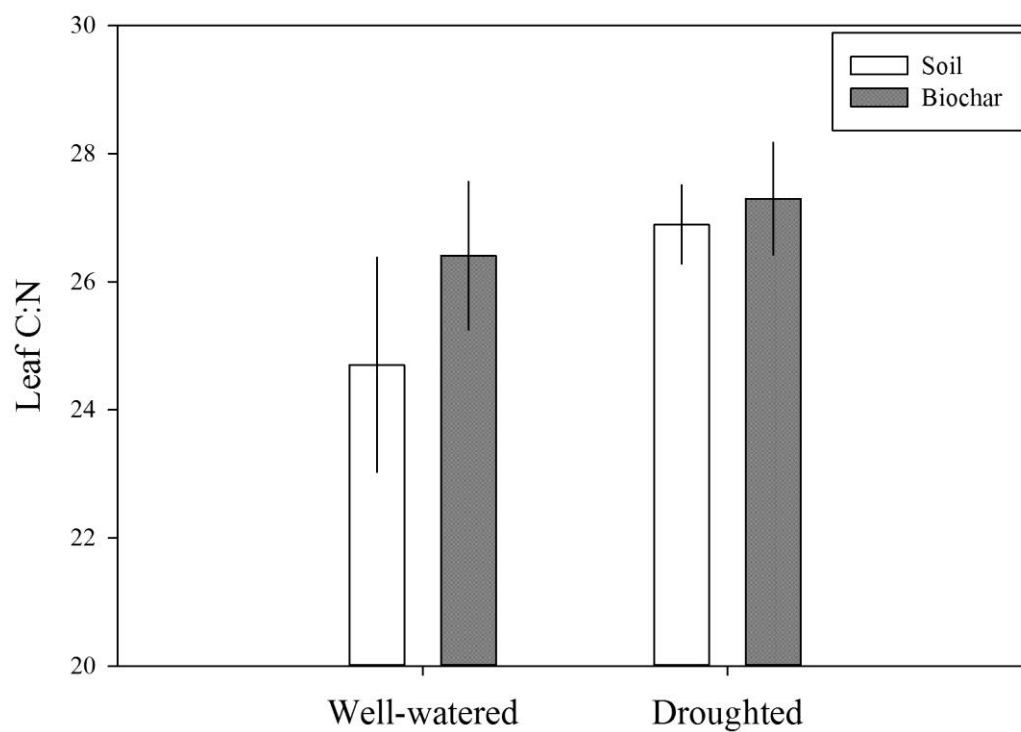


Figure 10. Leaf C:N ratio of giant miscanthus leaves at time of harvest. Bars are mean \pm se. Sample sizes ranged from 7 to 11.

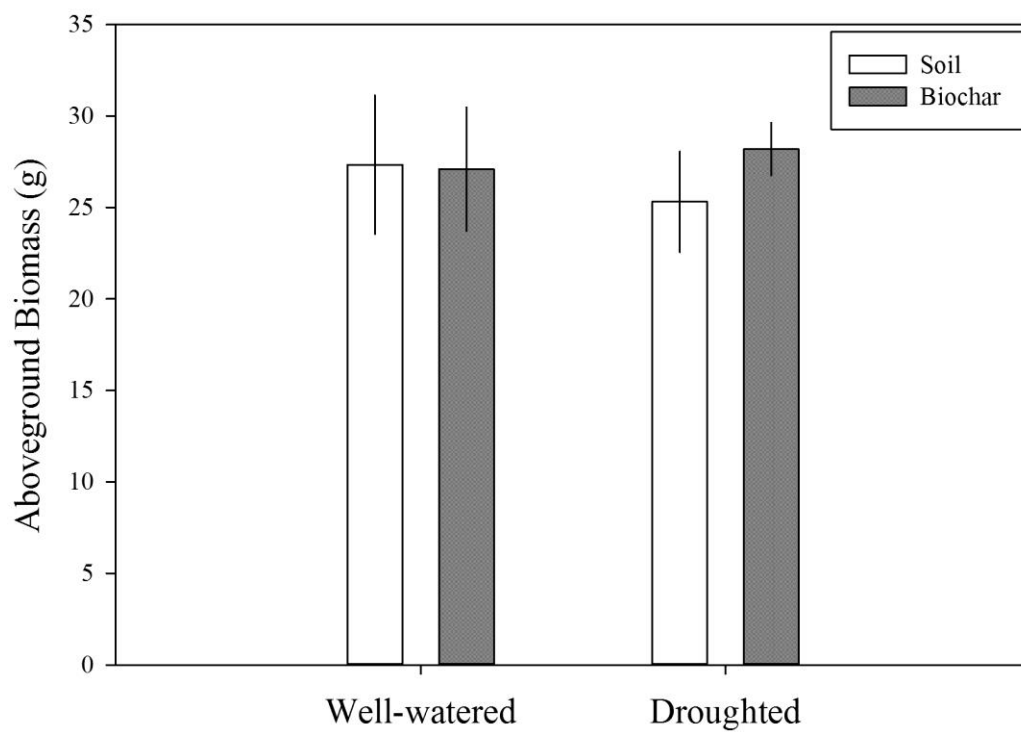


Figure 11. Total aboveground biomass of giant miscanthus at time of harvest. Bars are mean \pm se. Sample sizes ranged from 7 to 11.

Discussion

Water availability is one of the most limiting factors in crop production worldwide and is projected to become scarcer in the future with climate change (Dai, 2011). Low water availability can lead to drought stress in plants, which can reduce photosynthetic efficiency and ultimately cause lower yields (Ghannoum, 2009). Low quality soils are often utilized for bioenergy crop production in order to avoid competing with high quality land devoted to food crops (Ra *et al.*, 2012). These marginal soils can often have a lower water holding capacity than richer, agricultural soils, due to lower organic matter and higher bulk density. It is therefore important to understand how much crops, including giant miscanthus, will be affected by limited water availability due to either decreased precipitation or increasing stochastic distribution of rainfall. Periods of abundant rainfall interspersed with longer periods without rainfall may even lead to decreased net primary productivity compared to more frequent rainfall events even if the total rainfall received is the same (Knapp *et al.*, 2002).

Drought responses of C₄ plants can vary depending on the severity and length of the drought as well as the biochemical subtype of C₄ photosynthesis (Ghannoum, 2009). Previous studies have suggested that despite its C₄ nature, giant miscanthus is still at risk for yield loss due to drought (Price *et al.*, 2004; Richter *et al.*, 2008). Although there are now several studies detailing the physiological responses of giant miscanthus to varying degrees of water stress which were consistent with the findings in the present study (Weng, 1993; Clifton-Brown & Lewandowski, 2000; Ings *et al.*, 2013), few studies have documented the ability of giant miscanthus to recover from drought.

In the present study, the first physiological responses to drought in giant miscanthus were decreases in leaf water potential, photosynthetic rates, stomatal conductance, and the C_i/C_a ratio. These responses all occurred nearly simultaneously about 12 days after drought was induced, despite soil volumetric water content significantly decreasing just three days after withholding water. Photosynthetic rates of giant miscanthus remained above 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$ until leaf water potential reached -1.7 MPa, at which point rates dropped precipitously to just above 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Stomatal conductance decreased from pre-drought rates of 0.237 ± 0.0063 to $0.0623 \pm 0.0094 \text{ mol m}^{-2} \text{s}^{-1}$ at the

peak of drought stress. These results indicate that giant miscanthus was able to maintain substantial carbon gain even when under severe drought. The drop in the C_i/C_a ratio suggests that much of this limitation to photosynthesis was due to diffusional restrictions on the uptake of CO_2 , most likely as a result of stomatal closure (Ghannoum, 2009).

In a previous study, Weng (1993) did not see significant declines in photosynthetic rates until leaf water potential reached -1.3 to -1.5 MPa. Clifton-Brown *et al.* (2002) also found that leaf water potential of giant miscanthus receiving reduced irrigation decreased to -1.5 MPa, which drastically reduced photosynthetic rates and stomatal conductance. It should be noted that both of these studies utilized mid-day water potential measurements, whereas I measured predawn leaf water potential. Using predawn measurements is a best estimate of the maximum water status of the plant as drought proceeds as it closely, but not exactly, reflects soil water potentials (Donovan *et al.*, 2003). Because stomatal conductance was greatly reduced by drought in this study, mid-day leaf water potentials were likely lower than pre-dawn leaf water potentials, so the level of stress in my study was at least comparable, or perhaps even more severe than in the studies cited above. I found photosynthetic rates began to decline when leaf water potential had reached only -0.2 MPa, which suggests that productivities can be affected even by moderate drought. The drying rates in my study were also slower compared to Weng (1993) and more representative of rates that might occur in the field. Rapid drying rates in pot studies are not only unrealistic but can damage the photosynthetic apparatus more easily than if drying occurs more slowly.

It has been suggested that giant miscanthus keeps its stomata open longer under drought stress when compared to other *Miscanthus* species (Clifton-Brown & Lewandowski, 2000). For example, both Ings *et al.* (2013) and I found that stomatal conductance did not respond until after 12 days of drought; and in my study, photosynthesis and stomatal conductance had nearly simultaneous declines. This was also reflected in the decline in the C_i/C_a ratio, which suggests that much of the limitation to carbon gain was due to diffusional limitations caused by stomatal closure. There appears to be a large threshold before stomatal closure becomes significant, which suggests that giant

miscanthus may prioritize carbon uptake at the expense of maintaining higher plant water status. If droughts are of short duration, keeping stomata open could maximize productivity of this plant, whereas if droughts persist for longer periods of time, subsequent stomatal closure induced by low leaf water potentials could result in decreased uptake of CO₂ and lower rates of photosynthesis, which was the pattern seen in my study. The drop in the C_i/C_a ratio coincided closely with the timing of stomatal closure and suggests that much of the limitation imposed by drought on photosynthetic carbon gain is diffusional, although I did not explicitly test for biochemical limitations (Ghannoum, 2009). The lack of any substantial impact on Fv/Fm would support the notion that the capacity of PSII was only minimally affected, thus supporting the hypothesis that diffusional limitations predominated in this particular experiment. Certainly the rapid recovery upon rewatering adds to the argument that there was minimal damage to PSII with this short-term drought exposure.

Because chlorophyll fluorescence is a measurement of the maximum efficiency of photosystem II and often used as a proxy for whether a plant is experiencing photosynthetic stress (Murchie & Lawson, 2013), I expected to see a significant decrease in Fv/Fm of drought stressed plants that coincided with decreases in photosynthetic rates and stomatal conductance; however, there appeared to be little or no change in Fv/Fm over the course of this experiment. There did appear to be a lag response in Fv/Fm, which dropped the day after rewatering, possibly due to damage to the D1 protein, which is linked to the photosystem II core (Giardi *et al.*, 1996; Pieters & El Souki, 2005). It is likely I rewatered before any permanent damage could occur, which would explain why recovery of Fv/Fm occurred within two days after the significant decline.

I found that most physiological parameters of giant miscanthus were able to recover within one to three days of rewatering, suggesting that the photosynthetic mechanisms remain unharmed and that giant miscanthus is capable of tolerating at least a one-time short-term drought (Ghannoum, 2009). Zegada-Lizarazu and Monti (2013) found that *Sorghum bicolor* was also capable of recovering all photosynthetic capacity and chlorophyll fluorescence upon rewatering after a short-term drought. Another study by Medeiros *et al.* (2013) found that sugarcane varieties that were slow to respond to

drought and show any physiological changes were able to recover sooner after rewatering than those that responded more quickly to drought. These studies all support the hypothesis that photosynthetic mechanisms remained unharmed during short-term drought and that the inhibition of photosynthesis is due to stomatal closure rather than degradation of the photosynthetic complexes.

A decrease in chlorophyll content of plants under prolonged drought could be expected due to either reduced nitrate assimilation or protein degradation as the result of leaf senescence, but these mechanisms are poorly understood in C₄ plants subject to drought (Ghannoum, 2009). Medeiros *et al.* (2013) found that a short-term drought significantly decreased the chlorophyll content of sugarcane, a closely related C₄ plant, but not carotenoid content, whereas I found no differences in either chlorophyll or carotenoid content after drought or between soil treatments. I also found no differences between treatments in leaf C:N or specific leaf weight, even though specific leaf weight has been found to increase under drought stress in some species (da Costa & Cothren, 2011; Qaderi *et al.*, 2012), which results in an increased water use efficiency by decreasing the surface area to volume ratio.

Aboveground biomass of giant miscanthus also exhibited no significant differences between either watering regimes or biochar. This result is notable for two reasons. First, it demonstrates that despite being subjected to drought stress for three weeks, giant miscanthus did not incur significant losses in yield because few leaves were senesced, and those leaves that did senesce did not drop off and were therefore still accounted for in the harvest. Secondly, this result demonstrates that biochar at the rate applied had neither positive or negative impacts on the growth of giant miscanthus.

Previous studies have found increased above- and below-ground biomass with biochar addition (Kammann *et al.*, 2011; Vaccari *et al.*, 2011). Biochar has also been documented to benefit plant growth by increasing leaf area and plant height (Graber *et al.*, 2010) or by increasing leaf N content (Kammann *et al.*, 2011). I found no direct evidence that biochar had any effect on the growth or physiology of giant miscanthus, which may be due to a lower application rate being used compared to other studies. I chose an application rate near the average of previous studies, 50 t ha⁻¹, and a

review of the literature shows that application rates can vary widely. Jeffery *et al.* (2011) found the greatest increase in crop yield using an application rate of 100 t ha⁻¹, whereas Liu *et al.* (2013) reported that the majority of biochar studies utilize an application rate less than 30 t ha⁻¹ with an average increase in crop productivity of 11%.

Biochar, at the rate applied in this study, was able to increase the water holding capacity and decrease the bulk density of my soils, which should make these soils more favorable for plant growth. It is well known that biochar addition in soils decreases the bulk density (Vaccari *et al.*, 2011; Case *et al.*, 2012; Zhang *et al.*, 2012), which would therefore increase pore space available for water. Biochar has also been shown to increase water holding capacity of the soils at various application rates and different soil matric potentials (Brockhoff *et al.*, 2010; Laird *et al.*, 2010; Karhu *et al.*, 2011) likely due to the decrease in bulk density and the porous structure of biochar. These results, however, can drastically vary depending on the source material and temperature used in the creation of biochar, both of which can alter the physical and chemical properties of the biochar (Spokas *et al.*, 2012).

One important finding of this study was that volumetric water content of biochar-amended soils recovered much more quickly than that for soil-only treatments. The drying of the soils likely altered soil structure in such a way (perhaps by reducing the pore space available for water because soil particles compacted upon drying) that control soils were not able to recover their water status as fully as before drought was imposed. This result would likely become more apparent in an experiment featuring repeated drying and rewatering cycles and suggests that prevention of this phenomenon might be one of the more beneficial impacts of biochar amendment.

While several studies have documented the physiological effects of low water availability or drought stress on giant miscanthus, this is the first study that has also documented the recovery of physiological parameters in giant miscanthus. My results suggest that giant miscanthus is able to tolerate a short-term drought and is capable of making a quick and near full recovery with little to no effects on aboveground yield; therefore substantial growth might be achieved in areas with moderate drought occurrences. Future studies should focus on determining the amount of genetic variation in

giant miscanthus with respect to drought tolerance, and whether this could form the basis for crop improvement program to enhance yields in drought prone areas.

I was also unable to see any effect of biochar on the growth or physiological of giant miscanthus in the present study, which could be due to several factors: there may not have been enough time to observe a growth effect and/or the level of biochar application may not have been high enough to elicit a response. Given that the level I used is greater than most studies (Liu *et al.*, 2013), this suggests that giant miscanthus is relatively insensitive to the presence of biochar, and the slight enhancement of VWC that biochar does provide is not enough to elicit a stimulation of growth. My own field research (see previous chapter) supports this conclusion, as I could find no stimulation of growth in the field at application rates of 15 t ha⁻¹. Further research must be conducted to better elucidate the effects of biochar on plant physiology.

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Vita

Alyssa Leigh Teat was born in 1988 in Charlotte, North Carolina. She attended the University of North Carolina at Asheville from 2006 to 2011. During her time at UNCA, she worked on an undergraduate research project with Dr. Jonathan Horton that focused on the physiology of American Ginseng. Alyssa also participated in the NSF funded PIRE program at the University of Georgia in the spring and summer of 2010, where she studied genetic markers of invasive plants from both the US and China, and traveled throughout China and Tibet for 8 weeks. She was awarded a Bachelor of Science degree in Biology with a concentration in Cellular and Molecular biology in May 2011. From 2011 to 2014 Alyssa attended Appalachian State University, where she was awarded a GRAM fellowship for the 2012-2013 academic year, served as the Biology Graduate Student Association president, and was inducted into the Cratis D. Williams Society of Outstanding Graduates in 2014. Alyssa was awarded a Master of Science degree in Biology, with a concentration in Ecology and Evolutionary Biology, in May 2014.

